# A Microarray Format for Multi-Parameter Blood Group Serology

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## ABSTRACT

Microarray technology is continuously finding new applications in gene and protein profiling studies, and has huge potential for clinical and diagnostic applications. In this thesis multiple factors affecting probe - ligand interactions in a microarray format were investigated using blood group antigen and antibody sets for the development of a microarray based serology platform. Multiplexed microarray formats facilitate the complex analysis of structurally and functionally highly variable blood group antigens, which are classified into blood group systems. Epitopes often differ by a single sugar or amino acid substitution and the number of antigenic sites on red blood cells (RBC) and epitope accessibility vary significantly.

Initial experiments focused on multiple parameters affecting immobilisation and functionality of monoclonal and polyclonal antibodies on various microarray surfaces. A range of critical parameters affecting protein - ligand interactions, such as efficient blocking of non-specific binding, detergent type, RBC concentration, reaction volume and mixing, were optimised in a series of experiments with labelled anti-species antibodies. Selected microarray surface and optimised spotting and reaction conditions were then used for studies of antibody – RBC antigen interactions *in situ*.

Protein microarrays most frequently utilise classical antigen - antibody interactions where probe molecules are immobilised and target molecules are free in solution. A 'dual' solid-phase approach was investigated for blood group serology reactions where probe antibodies were immobilised on the microarray and target antigens were

carried on the RBC surface, which can be considered as the second solid-phase. This multiplex approach contrasts with the majority of current blood testing, which is generally detected by haemagglutination. This thesis provides the first comprehensive study of non-agglutination blood group determination in an open-plan microarray format. Using direct or indirect fluorescent labelling, both carbohydrate (ABO) and protein blood group antigens (Rhesus D, c, E and K) were successfully typed. The use of further antibodies for $Fy^a$ and $Fy^b$ typing, and the detection of RBCs sensitised with IgG have also been investigated using a microarray approach.

In order to verify the performance of diagnostic antibodies and quantitatively characterise their interactions in a label-free detection system, we have exploited the Biacore system as a recognised platform for real-time interaction studies. Although this system is not designed for the high throughput applications needed in blood testing, in this study it provided detailed information on the interactions between blood group A antibody and RBCs of different specificity. In addition, immobilisation of synthetic carbohydrate antigen on a Biacore chip permitted the quantitative determination of the reactivity of monoclonal antibodies in a 'reverse typing' system.

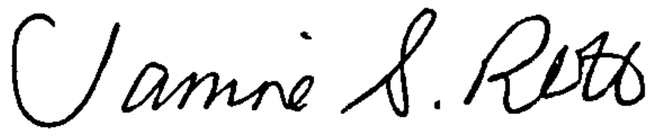
Reverse typing for the detection of antibodies against blood group antigens is an integral part of blood group serology. Biacore experiments investigated direct attachment of synthetic RBC antigens onto the solid-phase, and subsequent detection using antibodies. However, native multi-transmembrane RBC antigens are not

optimal for microarray immobilisation. It is known that whole RBC ghosts can be immobilised in macroassays, but this approach was considered to be less suitable for miniaturised microarray probe spots due to cell size and stability. In this study an alternative method of RBC membrane fragment spotting was used for the first time for blood group serology by employing novel surfaces and defining parameters suitable for a reverse reaction system.

In summary this investigation has 1) established a microarray format and the reaction conditions for antibody - antigen interaction studies in blood group serology; 2) for the first time successfully exploited microarray format for comprehensive blood typing; 3) examined the novel technique of membrane fragment microarray immobilisation for a reverse, antibody screen reaction; 4) verified the findings and quantitatively characterised the interactions on Biacore, a surface plasmon resonance real-time detection system. This provides a strong basis for the development of microarray based multi-parameter blood group serology diagnostic platforms.

## DECLARATION

Except where specific reference is made to other sources, the work contained in this thesis is the original work of the author. It has not been submitted in whole or in part, for any other degree.

Signed 

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Janine S. Robb.

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# ABBREVIATIONS

| | |
|---|---|
| $\mu$l | microlitre |
| $\mu$m | micrometre |
| 3-D | three-dimensional |
| Ab | antibody |
| Ag | antigen |
| BIA | biospecific interaction analysis |
| bmp | bitmap |
| BSA | bovine serum albumin |
| cDNA | complementary DNA |
| Conc. | concentration |
| DNA | deoxyribonucleic acid |
| ELISA | enzyme-linked-immunosorbant assay |
| FCS | foetal calf serum |
| FITC | fluorescein isothiocyanate |
| gIgG | goat immunoglobulin G |
| hIgG | human immunoglobulin G |
| hIgM | human immunoglobulin M |
| HIV | human immunodeficiency virus |
| HSA | human serum albumin |
| ID | identification |
| IgG | immunoglobulin G |
| IgM | immunoglobulin M |
| IVD | *in vitro* diagnostic (medical devices) |
| LISS | low ionic strength saline |
| MALDI | matrix assisted laser desorption/ionisation |
| MAb | monoclonal antibody |
| mIgG | mouse immunoglobulin G |
| mIgM | mouse immunoglobulin M |
| mm | millimetre |
| min | minute |

| | |
|---|---|
| mRNA | messenger RNA |
| MS | mass spectrometry |
| NAT | nucleic acid amplification techniques/testing |
| nl | nanolitre |
| NSB | non-specific binding |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| RBC | red blood cell (erythrocyte) |
| RCA | rolling circle amplification |
| reps | replicates |
| RhD | Rhesus D |
| rIgG | rabbit immunoglobulin G |
| RI | refractive index |
| RNA | ribonucleic acid |
| RU | response units |
| S/N | signal to noise ratio |
| scFv | single chain variable region |
| SDS-PAGE | sodium dodecyl sulphate-polyacrylamide gel electrophoresis |
| SELDI | surface enhanced laser desorption/ionisation |
| sIgG | sheep immunoglobulin G |
| SCGTI | Scottish Centre for Genomic Technology and Informatics |
| SNBTS | Scottish National Blood Transfusion Service |
| SPR | surface plasmon resonance |
| tif | tagged image file |
| TOF | time of flight |

# CHAPTER 1

# INTRODUCTION

# 1. INTRODUCTION

The transfusion of blood and blood products can save lives or greatly improve the quality of life for recipients. Donor blood products must be compatible with the recipient's blood system or this life saving treatment can result in severe, if not fatal, consequences. The testing of samples for blood transfusion purposes is consequently a highly regulated area.

The area of blood transfusion testing is covered by both United Kingdom (U.K.) and European law in the U.K., and by other regional quality systems worldwide. Products for use in blood testing must be manufactured and standardised in accordance with the Guidelines for the Blood Transfusion Services in the United Kingdom (U.K. Blood Transfusion Services, 2002) and comply with the European Union *in vitro* Diagnostic Medical Devices Directive (IVD Directive (98/79/EC), (European Parliament website). Products for use in ABO, RhD typing and antibody screening are covered by Annex 2, List A and List B of the IVD Directive, which cover the most critical blood testing products. In the United States of America, blood testing products must comply with Food and Drug Administration (FDA) regulations. Testing procedures must comply with U.K. Blood Transfusion Service Guidelines and the Guidelines for compatibility procedures in blood transfusion laboratories (Working Party of the British Committee for Standards in Haematology Blood Transfusion Task Force, 2004).

The purpose of blood testing of both donors and patients is to ensure as far as possible the provision of safe products for blood transfusion. A safe product is one

that is blood type compatible and that is free of known pathogenic organisms, to which there is a test available.

In order to provide compatible blood, both donors and recipients are tested for their blood type and for the presence of blood group antibodies. These serological tests on blood samples are mandatory and involve the assay and interpretation of antibody-antigen interactions. This involves the complex analysis of structurally and functionally highly variable blood group antigens, which are classified into blood group systems. Epitopes often differ by a single sugar or amino acid substitution and the number of antigenic sites on erythrocytes (red blood cells) and epitope accessibility vary significantly. Blood group antigens are discussed in more detail in section 1.2.

ABO is the most important of the blood group systems, due to the presence of alloantibodies to the antigens of the system that the individual lacks. For example, if a group A or O patient was given group B blood, then the anti-B in their plasma would react with the B antigen on the donor red cells and cause a severe or fatal transfusion reaction. The Rhesus blood group system is second in importance only to the ABO system. This system is clinically significant due to the high immunogenicity of the blood group antigens within this system. The transfusion of 500 ml of Rhesus D (RhD) positive blood to an RhD negative individual is likely to result in the production of an anti-D in up to 81.8 % of cases (Pollack *et al.*, 1971). The production and presence of alloantibodies has implications in subsequent transfusions.

Current testing methods will be described in this chapter, but it is hoped that a novel multiplexed microarray format would facilitate improvements to the existing technology. In this thesis multiple factors affecting probe-ligand interactions in a microarray format were investigated using blood group antigen and antibody sets for the development of a novel solid-phase non-agglutination based microarray serology platform.

The primary aim of this project was to study the parameters important for protein-ligand interactions in a microarray format, and to apply the optimised parameters to develop blood typing and erythrocyte antibody screening in a solid-phase microarray format.

## 1.1 Blood Grouping Related Protein and Ligands: Structures and Interactions

In this project, the interaction of blood group related antigens and antibodies was studied using a microarray format. Antibodies are glycoproteins, and blood group related antigens are either proteins or carbohydrates.

### 1.1.1 Protein Structure - General

Proteins are made up of amino acids forming long polypeptide chains (see e.g. Berg *et al.*, 2002). The carboxyl group of one amino acid joins to the amino group of another to form a peptide bond. The primary structure of proteins describes the linear sequence of amino acids. The secondary structure of peptides and proteins involves the organisation into structures such as $\alpha$ helix or $\beta$ pleated sheets, which are

stabilised by hydrogen bonds. The placement of the disulphide bonds determines the secondary structure. Disulphide bonds between cysteine amino acid residues on the chain stabilise the peptide chain. Tertiary structure describes the three-dimensional (3-D) structure of the complete protein where the relationship of amino acids to one another is very different than in the primary structure. Steric relationships can affect the structure. The tertiary structure can alter if subjected to small changes in the environment. The quaternary structure consists of the conformation of all subunits of a functional protein stabilised by electrostatic and hydrogen bonds. Proteins may also contain molecules of a non-amino acid source, such as vitamins, minerals, lipids and carbohydrates. Borrebaeck *et al.* (2001) found that proteins may denature and lose their tertiary and quaternary structure when attached to a solid surface such as those used in microarrays, and therefore care must be taken to retain their structure and function.

### 1.1.2 The Immune Response - Antibody Production and Structure

The transfusion of blood impacts on the immune response of recipients. To minimise the immunological complications of transfusion, a number of procedures are used in both the procurement and provision of blood and blood components (U.K. Blood Transfusion Services, 2002; Working Party of the British Committee for Standards in Haematology Blood Transfusion Task Force, 2004). Blood donor recruitment procedures, blood typing and blood processing such as leucocyte depletion, gamma irradiation or washing have been improved and refined to minimise these risks (see review by Brand, 2000).

The immune system has evolved to combat infectious and non-self agents, by distinguishing non-self molecules from self, which if left could potentially kill the host (see e.g. Roitt *et al.*, 1996). There are two types of immune response, the difference being in whether the action is non-specific or specific. An innate (non-adaptive) immune response involves the non-specific interaction of phagocytic cells that bind, internalise and kill pathogens, or the action of lysozymes and natural killer cells (see e.g. Harlow and Lane, 1988). Innate immunity is a residual element of the primitive immune system, whereas the specific immune response system, or adaptive response, is more highly evolved. The adaptive immune response gives improved immunity on each encounter with a pathogen, or non-self antigen, as it remembers the immunogen and rapidly responds to another encounter with identical or closely related antigen.

The immune system uses two systems in parallel; the humoral and cellular immune response systems. In the humoral system, antibodies function as recognition elements and bind to foreign molecules (see e.g. Berg *et al.*, 2002). In the cellular system, killer T cells kill other cells that display foreign antigens.

Helper T lymphocytes aid both systems by stimulating the differentiation and proliferation of B-lymphocytes (antibody producing cells) and killer T cells. Other molecules such as cytokines assist in the immune response by signalling between cells. Cytokines are proteins or peptides and can be further classified into interferons, interleukins and colony stimulating factors.

Antibody molecules are secreted by plasma cells, which are derived from B-lymphocytes, and are produced by an individual in response to the presence of a foreign 'antigen' in the body (Roitt *et al.*, 1996). An antigen may be any kind of molecule that is recognised as foreign to the body e.g. infectious agents or constituents of blood component transfusion. A specific area of the antigen known as the antigenic determinant, or epitope, determines the specificity of the antibody. There is great diversity in antibody generation to create a repertoire of specificities. This diversity is possible due to multiple variable region genes in germ lines, somatic mutation, random pairing of heavy and light chains and variation in gene recombination (review, Harlow and Lane, 1988).

Antibodies (Ab), or immunoglobulins (Ig), may be divided into the following classes (or isotypes) depending on structure: IgA, IgD, IgE, IgG or IgM, and further details are shown in **Table 1.1.** In blood testing alloantibodies of type IgG and IgM are most commonly encountered. Structurally, antibody molecules are $\beta$ pleated sheets, with anti-parallel strands, linked by disulphide bonds. The structure of IgG and IgM molecules are discussed now in more detail.

### 1.1.2.1 IgG Antibody Structure

Most blood group alloantibodies are of the IgG isotype. An IgG molecule is made up of four polypeptide chains; two identical heavy chains and two identical light chains. The heavy chains are each of approximately 50 kDa and the light chains 25 kDa, therefore the entire IgG molecule is approximately 150 kDa, depending on subclass (see **Table 1.1**).

**Table 1.1.** Classes of antibodies; heavy chain structure, valency, molecular weight, carbohydrate content and function (from Roitt *et al.*, 1996; Harlow and Lane, 1988 and publications Feinstein *et al.*, 1971; Sarma *et al.*, 1971; Amzel and Poljak, 1979).

| Class | $IgG_1$ | $IgG_2$ | $IgG_3$ | $IgG_4$ | IgM | IgA | IgE | IgD |
|---|---|---|---|---|---|---|---|---|
| Heavy chain | $\gamma_1$ | $\gamma_2$ | $\gamma_3$ | $\gamma_4$ | $\mu$ | $\alpha$ | $\varepsilon$ | $\delta$ |
| Valency (no. of antigen-binding sites | 2 | 2 | 2 | 2 | 10 | 2,4 or 6 | 2 | 2 |
| Molecular weight (kDa) | 146 | 146 | 170 | 146 | 970 | 160 (basic) | 188 | 184 |
| Carbohydrate content (%) | 2-3 | 2-3 | 2-3 | 2-3 | 12 | 7-11 | 12 | 9-14 |
| Function | Secondary immune response | Secondary immune response | Secondary immune response | Secondary immune response | Primary immune response | Protects mucous membranes | Protects against parasites, associated with allergic response | Unknown (may be involved in lymphocyte differentiation |

Blood group alloantibodies are most frequently IgG, and usually $IgG_1$ or $IgG_3$, or both in some cases. Each of the chains folds into structural domains of approximately 110 amino acids in length. Each light chain consists of a variable domain ($V_L$) and a constant domain ($C_L$). Each heavy chain consists of one variable domain ($V_H$) and three constant domains ($C_H1$, $C_H2$ and $C_H3$). The variable domains of both heavy and light chains associate to form the antigen-binding site, of which the IgG molecule has two. Structural studies performed by Sarma *et al.* (1971) determined the maximum distance between the two antigen-binding sites is 14 nm. Disulphide bonds at the $C_L$ and the $C_H1$ domains link the heavy and light chains (early structural work performed by Feinstein *et al.*, 1971; Sarma *et al.*, 1971). There is a flexible region between the $C_H1$ and $C_H2$ domains, which is known as the hinge, and here the two heavy chains are linked by disulphide bonds. The hinge region allows flexibility in the molecule, and thus allows the two antigen binding sites to operate independently. The $C_H3$ domains link with each other by non-covalent interactions. The structure of an IgG molecule is shown schematically in **Figure 1.1.**

Using appropriate concentrations and time, the enzyme papain can cleave an IgG molecule into three fragments (from which identifying nomenclature is derived): two identical Fab fragments (fragment antigen-binding) and one Fc fragment (fragment crystalline) because it can be crystallised (Porter, 1959). The Fc fragment has specific placental transmission sites and a site for binding complement (Berg *et al.*, 2002).

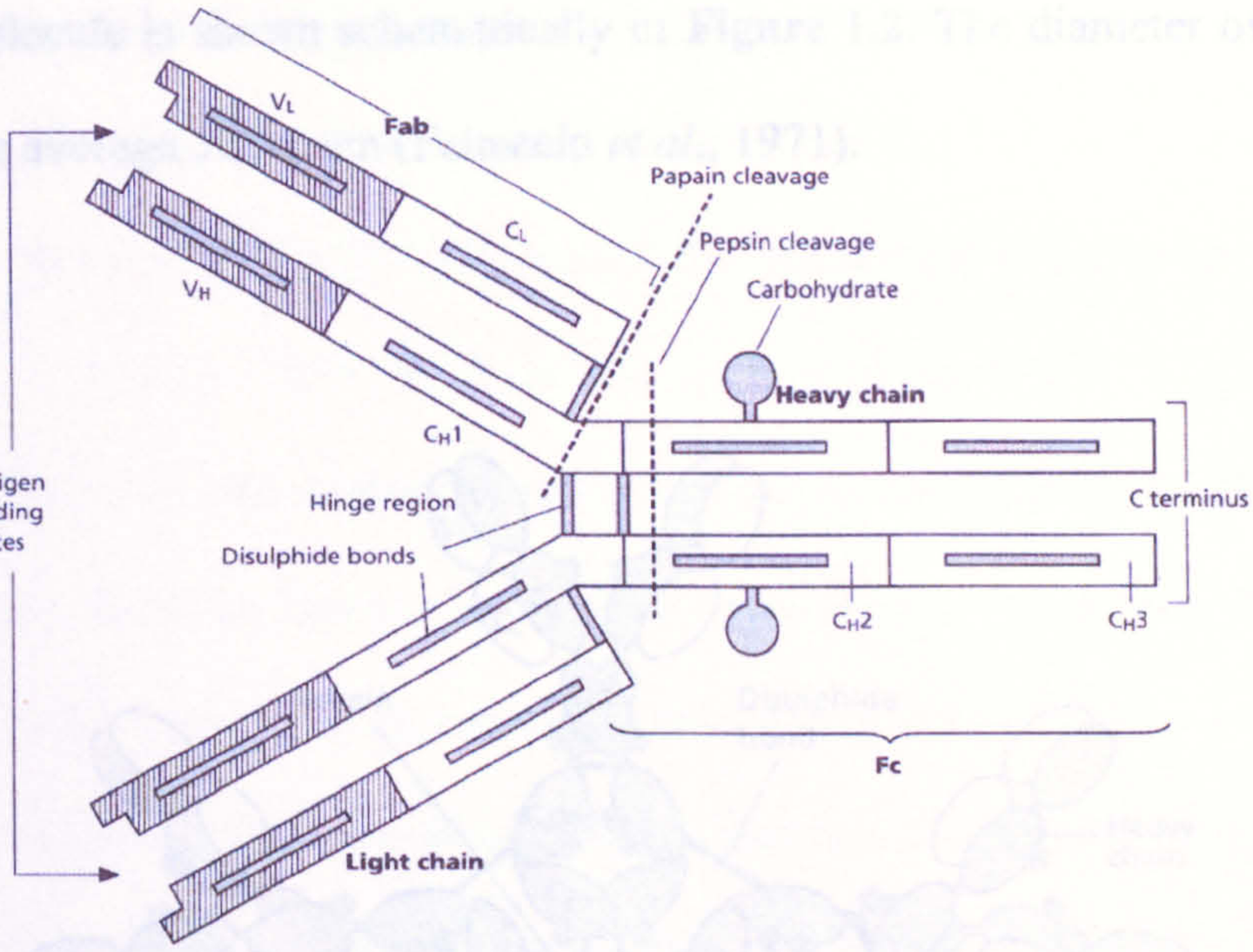



**Figure 1.1**. The structure of an IgG molecule, showing locations of domains, disulphide bonds and enzyme cleavage points (picture from Mollison *et al.*, 1997).

Within each of the variable domains of the heavy and light chain there are three hypervariable regions of approximately seven to twelve amino acids. The hypervariable regions lie within 'framework' regions in which there is little variation. The hypervariable regions, also known as complementarity determining regions (CDRs), form loops at the tip of the Fab structure and make up the antigen-binding site. It is hypervariability at these regions that gives the diversity of the specificity of antibody molecules (see e.g. Harlow and Lane, 1988).

### 1.1.2.2 IgM Antibody Structure

Generally, the primary immune response initially produces IgM isotype antibodies, but over a period of time there is a transition to IgG antibodies. IgM molecules have a pentameric structure of the basic four-polypeptide chain described for IgG, therefore consisting of ten antigen-binding sites (Feinstein *et al.*, 1971). The structure

of an IgM molecule is shown schematically in **Figure 1.2**. The diameter of an IgM molecule is on average 30-31 nm (Feinstein *et al.*, 1971).

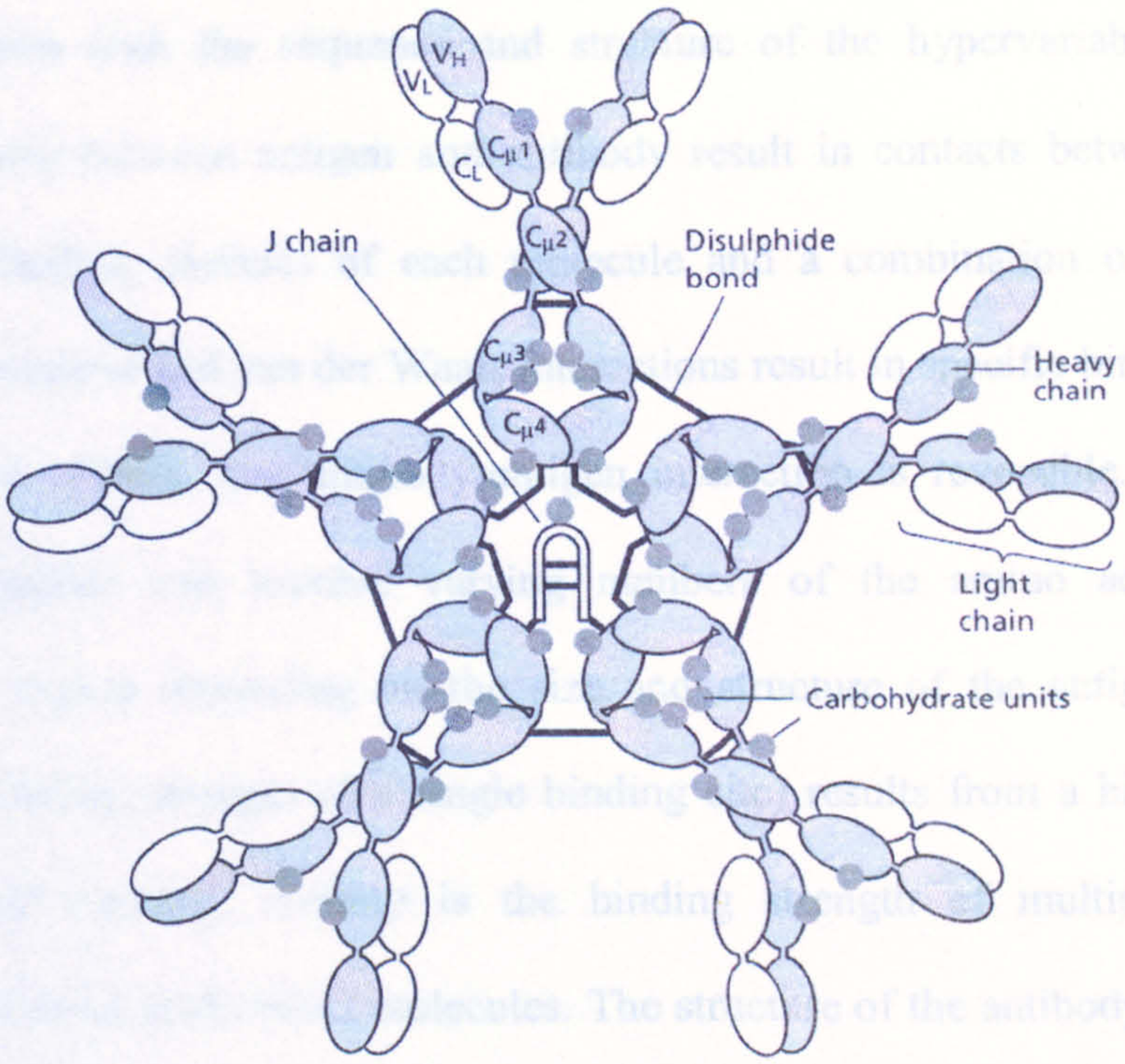



**Figure 1.2.** The structure of an IgM molecule, showing locations of domains, carbohydrate units, J chain and disulphide bonds (picture from Mollison *et al.*, 1997).

The type of heavy chain is described as $\mu$ and it differs from the $\lambda$ chain in that it has no hinge region but an extra constant domain $C_H4$. The antibody heavy chains are held together by disulphide bonds between the $C_H3$ domains. The IgM molecule also has a 'J' chain; a polypeptide chain that helps in the assembly of the molecule. IgM molecules do not have hinge regions but flexibility can occur around the $C_H2$ domains.

### 1.1.3 Structure and Interaction of Antibodies and Antigens

In every class of antibody, the $V_L$ and $V_H$ variable domains link at the ends of the arms of the antibody structure (Berg *et al.*, 2002) and the three hypervariable regions of each chain come together to form the antigen-binding site. The antibody specificity varies with the sequence and structure of the hypervariable regions. Complementarity between antigen and antibody result in contacts between amino acids at the binding surfaces of each molecule and a combination of hydrogen bonding, electrostatic and van der Waals interactions result in specific binding (from Mollison *et al.*, 1997). The antibody-antigen interaction is reversible. Antibody-antigen interactions can involve varying numbers of the amino acids at the hypervariable region depending on the size and structure of the antigen. Strong affinity (the binding strength of a single binding site) results from a high level of interaction and bonding. Avidity is the binding strength of multiple binding interactions between multivalent molecules. The structure of the antibody molecules used in haemagglutination assays determines how the interaction is detected. IgM molecules bind to form haemagglutination directly, while IgG molecules most often require the addition of a secondary antibody or other interaction to cause agglutination. IgG antibodies of some specificities can cause direct haemagglutination e.g. because of high antigen site density.

When using a solid surface to perform antibody-antigen interaction studies, haemagglutination is inhibited as one component of the test is not free in solution, but is immobilised. Therefore interactions have to be detected in other ways. Also of consideration is the accessibility of the antigen-binding sites of the antibody to the

antigen. When molecules are immobilised on a solid support orientation may be random and some of the antigen or antigen-binding sites may be inaccessible.

### 1.1.4 Antibody Production

Antibodies are used throughout the field of immunoassays, including blood typing diagnostics. The isolation of polyclonal antibodies or the manufacture of monoclonal antibodies is of high importance to ensure continuity of supply. Both antibody types are used in this project. While polyclonal antibody preparations are specific towards multiple epitopes of the same antigen, monoclonal antibodies are specific to a single epitope.

Polyclonal antibodies have been used in immunological testing for many years. These are alloantibodies produced in response to a foreign antigen and can be isolated for use in assays. Polyclonal antibodies can be produced in many animals but those used in blood grouping are usually of human source, or rabbit (e.g. current polyclonal anti-human IgG). Serum containing alloantibodies may have to be adsorbed to remove other unwanted antibodies, and because of this polyclonal antibodies are more time consuming and costly to produce than monoclonal derived antibodies. Batches of polyclonal antibodies can also be highly variable.

In 1975, Köhler and Milstein defined methods to produce antibodies of a 'predefined specificity'. These hybridoma cells can produce monoclonal antibodies (MAb) of single specificity in unlimited quantities. Fusing lymphocytes taken from an immunised donor species with myeloma cells produces hybridoma cell lines. A

myeloma cell has antibody-producing features but does not produce functional antibody (Harlow and Lane, 1988). It also undergoes continuous cell division providing the fused hybrid cell with immortality. Enhancement media are added to the lymphocytes and myeloma cells which allow and encourage the fusion of the cells. This can result in cells that express both the antibody of interest and the immortality of the myeloma cell. After fusion, cells are grown in selective media that eliminates growth of unfused cells (Köhler and Milstein, 1975). Fused cells are then assessed for specificity of antibody produced. Most ABO and Rhesus D antibodies used in blood typing are derived from monoclonal cell lines. There are some blood group antigen specificities, however, that have not been produced by hybridoma technology, despite several attempts. As the method is also fairly labour intensive, other methods have been developed.

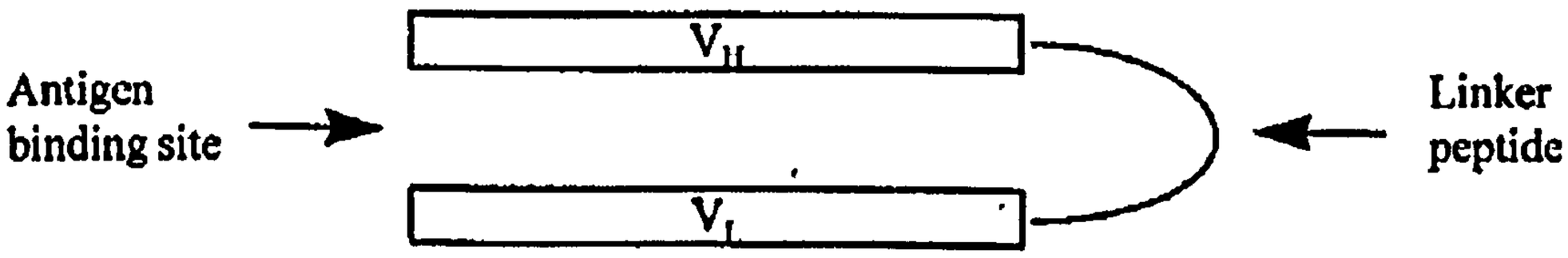



**Figure 1.3.** Structure of a single-chain variable fragment (scFv) (original reference Bird *et al.*, 1988, picture from Issitt and Anstee, 1998).

An alternative to poly and monoclonal antibodies are *in vitro* produced fragments of antibody molecules containing the antigen-binding site. This can be used in the production of antibody Fab or single chain Fv fragments (see **Figure 1.3**). When filamentous bacteriophage are used to express the molecule on their surface, this is commonly referred to as 'antibody phage-display'. This method concentrates on the isolation of the genes for the variable regions of the antibody. These can be placed in

a constant region construct by recombinant DNA techniques. This area developed rapidly in the early 1990s, and Hoogenboom and Winter (1992) and Winter *et al.* (review, 1994) describe the advances in the technology. In 1994, Hughes-Jones *et al.* described the characterisation of scFv to blood group antigens B, RhD, RhE, $Kp^b$ and HI, prepared by use of phage-antibody libraries. In 1995, Griffin and Ouwehand demonstrated the production of a human monoclonal antibody to platelet glycoprotein IIIa from a V-gene phage-display library. Marks and Marks (1996) reviewed the strategy of the phage library method for the preparation of clinically useful antibodies. Miescher *et al.* (1998) described the use of phage-display to isolate human Fab fragments with specificity to Rhesus D blood group antigen. Chang and Siegel (2001) successfully isolated human IgG anti-B from a Fab phage-display library. Phage-display libraries could potentially be used to identify antibody fragments for difficult to source blood typing reagents. Another method for the display of functional proteins is ribosome display, where both the protein and its mRNA remain attached to the ribosome. This technique has been used to produce scFv fragments (Hanes and Plückthun, 1997; Schaffitzel *et al*, 1999), which can be enriched to enhance binding properties.

### 1.1.5 Antigen Structure

An antigen can be described as any substance which when introduced to a foreign immune system can elicit the production of an antibody against it. Antibodies are used throughout this work, and the ligands/antigens they are directed against include plasma/serum proteins, antibodies, blood group determinants on erythrocytes, synthetic peptides and synthetic blood group determinants. Blood groups are

determined by the antigens on the erythrocyte surface and these antigens are either protein or carbohydrate structures which are discussed now in more detail.

## 1.2 Transfusion Related Antigens and Antibodies

### 1.2.1 Blood Group Antigenic Determinants and Their Structure

The primary focus of this work was to develop a novel system for the determination of blood type (ABO and RhD). The erythrocyte carries the blood group determinants, although for some groups these can be found in other secretions of the body.

Erythrocytes are developed from an erythroid progenitor cell line, and during their maturation lose their nucleus and all other genetic material. Erythrocytes have developed the specialised function of carrying oxygen around the body. Normal erythrocytes are described as biconcave discs, 7.8-8.3 $\mu$m in diameter and 1.7 $\mu$m thick (Lessin and Bessis, 1979). The membrane is made of proteins, lipids and carbohydrates (review, Schenkel-Brunner, 2000). The cell has great flexibility to enable it to pass through small capillaries of the body, and this property is due to the cell cytoskeleton – a network of proteins beneath the membrane and attached to the lipid bilayer (review, Gilligan and Bennett, 1993). The main component of the cytoskeleton is spectrin – a molecule of two subunits that combine to form heterodimers, which in turn form tetramers. These tetramers interact with the proteins actin and ankyrin to attach to the membrane through transmembrane proteins. A schematic of the erythrocyte membrane and associated proteins is shown in **Figure 1.4.**

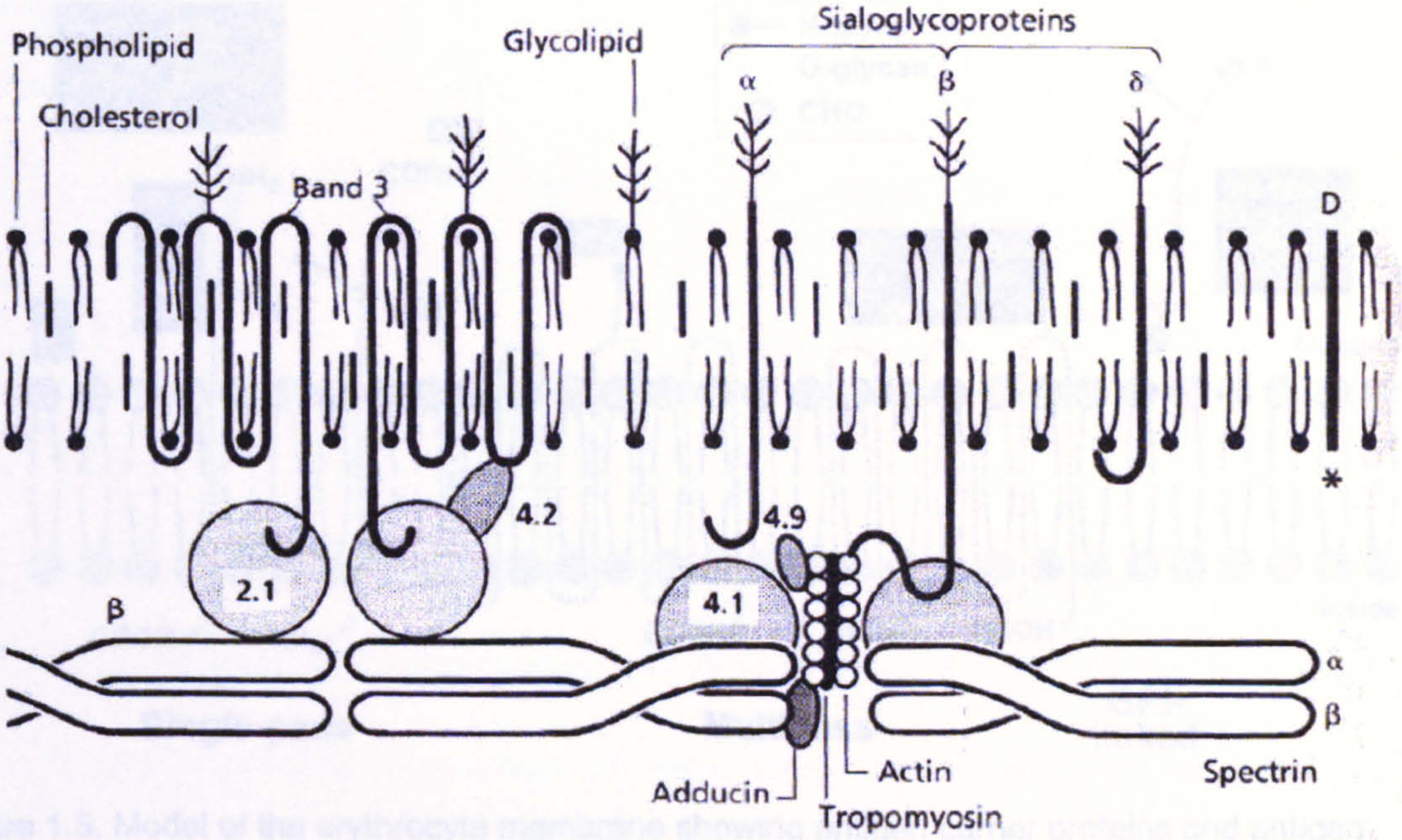



**Figure 1.4.** The erythrocyte membrane and associated proteins (picture from Mollison *et al.*, 1997).

Blood group antigens are either carbohydrates, single-pass membrane proteins, multi-pass membrane or glycosylphosphatidylinositol linked proteins (review, Reid and Lomas-Francis, 1997). Transmembrane proteins attach themselves to a layer of phospholipids in the membrane, and they can traverse through the membrane many times (see e.g. Mollison *et al.*, 1997). **Figure 1.5** shows a schematic of the erythrocyte antigens and their attachment to the cell.

A blood group consists of antigens produced by alleles at a single genetic locus (Issitt and Anstee, 1998). More than 270 blood group antigens (review, Daniels, 2002) have been placed into at least 29 blood group systems (Reid, 2003). ABO is the most clinically significant blood group system, followed by the blood group systems Rhesus, Kell, Duffy and Kidd.

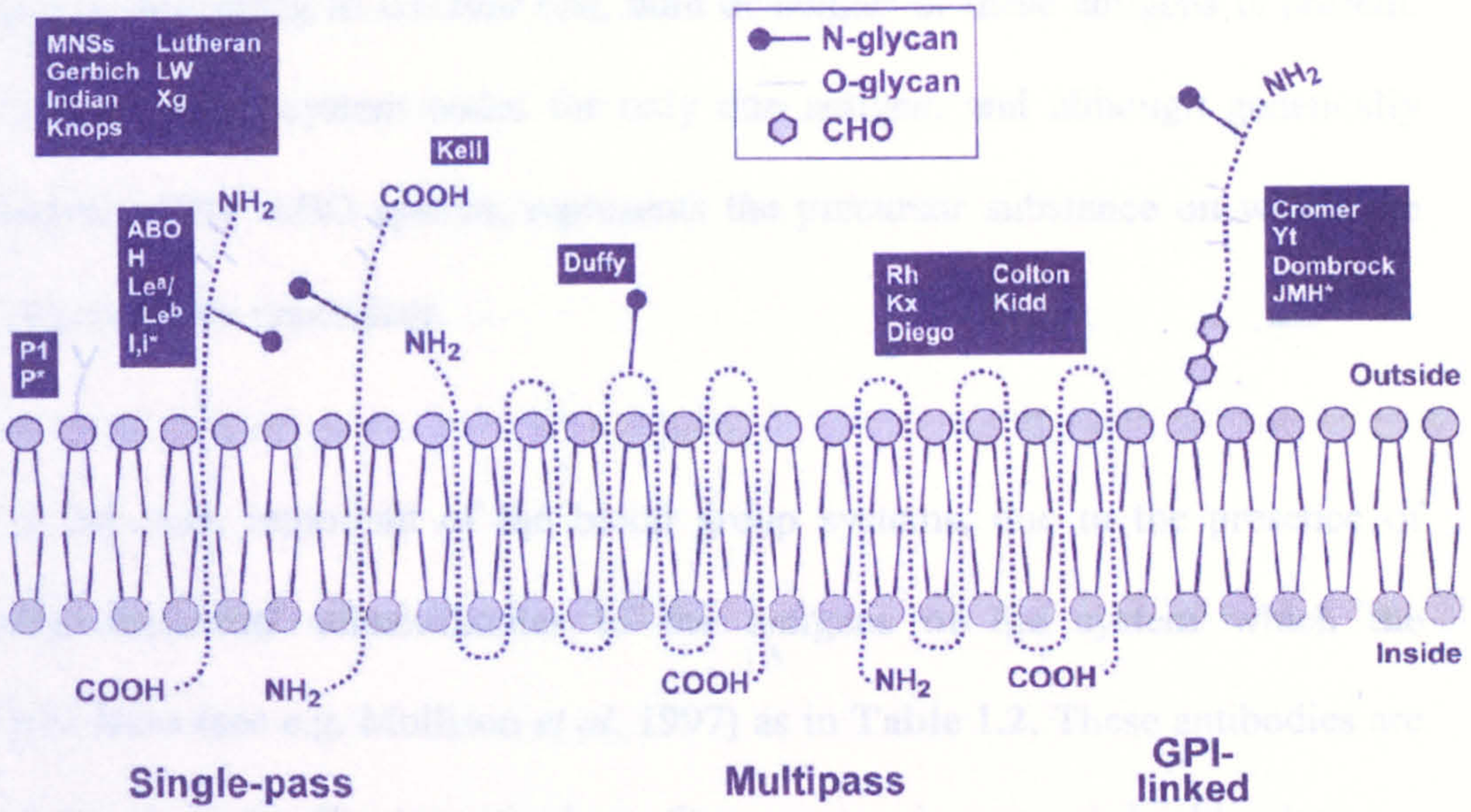



**Figure 1.5.** Model of the erythrocyte membrane showing antigen carrier proteins and antigen attachment (picture from Reid and Lomas-Francis, 1997).

Antibodies to antigens of the ABO, Rhesus and Kell systems were studied in this project, and these systems are discussed below. The Rhesus, MNS and Kell blood group systems are considered the most complex. The molecular mechanisms that generate the great diversity that exists in blood group antigens include point mutation, deletion, insertion, altered splicing events, intra- and intergenic crossover and gene conversion (Reid and Rios, 1999). Most antithetic blood group antigens arise from missense mutations (where the change of one nucleotide results in a change in encoded amino acid). Other clinically significant blood group systems are referred to in **Table 1.4**.

### 1.2.1.1 The ABO & H Blood Group Systems

Discovered in 1900 by Karl Landsteiner (Landsteiner, 1900 and 1901), the ABO blood group system is based on the presence or absence of two carbohydrate antigens, A and B (Morgan, 1960). It is possible to classify individuals into four

main groups according to whether one, both or neither of these antigens is present. The H blood group system codes for only one antigen, and although genetically independent of the ABO system, represents the precursor substance on which the ABO structures are dependent.

ABO is the most important of the blood group systems, due to the presence of 'naturally occurring' alloantibodies to the antigens of the system which the individual lacks (see e.g. Mollison *et al.* 1997) as in **Table 1.2.** These antibodies are referred to as 'naturally occurring' as they appear in normal healthy humans naturally within six months of birth. However, work performed by Springer *et al.* (1959) demonstrated that the production of these antibodies is in fact an immune response. Using chicks, they showed that germ-free chicks held in a sterile environment did not form anti-B, whereas conventional chicks produced anti-B within days of exposure to the normal environment.

**Table 1.2** Antigens and antibodies present in ABO blood groups.

| Blood group | Antigens present on RBC | Antibodies in plasma |
|---|---|---|
| A | A | Anti-B |
| B | B | Anti-A |
| AB | A and B | None |
| O | None | Anti-A and Anti-B |

Therefore, it is thought that ABO system alloantibodies are raised to substances in nature, which have similar structure to A and B antigens e.g. microbial surface antigens. These alloantibodies are mostly IgM, and when tested against appropriate erythrocytes, react directly without the use of a secondary detection antibody. Due to

the presence of these antibodies, serum/plasma samples should be tested to aid confirmation of the ABO group by testing the sample against known group A and B erythrocytes. ABO system antibodies are efficient at complement activation, meaning that incompatible cells will be caused to lyse quickly and lead to severe transfusion reactions.

The antigenic determinants of the ABO and H systems are carbohydrates and, therefore, are not direct gene products of the *A, B* and *H* genes (Morgan and Watkins, 1959; Morgan, 1960; Rege *et al.*, 1963). These genes code for glycosyltransferase enzymes (Watkins, 1966), which catalyse the transfer of individual sugar molecules (monosaccharides) from a donor substrate to a pre-determined precursor substance, to form oligosaccharides representing the A, B or H antigens.

Structural studies have been performed on the oligosaccharides present in the ABO and H blood groups and have been used to determine the structure of A, B and H determinants. From these investigations six types of determinants have been identified (see e.g. Schenkel-Brunner, 2000).

ABO and H carbohydrate chains are conjugated mostly to polypeptides to form glycoproteins (carbohydrate + protein) and attached to the anion exchange protein band 3 and the glucose transport protein band 4.5 (review, Daniels, 2002). There are approximately one million monomers of band 3 protein and 0.5 million monomers of band 4.5 protein per adult red cell. A smaller amount of chains are conjugated to ceramide to form glycolipids or glycosphingolipids (carbohydrate + lipid). The

average number of ABH sites on adult red cells is in the range of 1.5 – 2.0 x $10^6$ (Schenkel-Brunner, 2000). There are large differences in the density of antigen sites among different populations and blood groups of red cells. Economidou *et al.* (1967) gave results of an evaluation of the level of A and B antigens on different blood groups, and Cartron *et al.* (1974) reviewed group A subgroup antigen density. Voak and Lodge (1970) discussed the high number of $A_2B$ individuals found in certain populations and claim that this may be due to H substance deficiency which results in group $A_1B$ individuals appearing serologically as $A_2B$. Antigen site density is important in blood grouping as low levels of antigen may result in mis-grouping.

In Europe approximately 80 % of all group A individuals are $A_1$ and the remainder belong to subgroup $A_2$. There are qualitative differences between group $A_1$ and group $A_2$ in the types of precursor chains used, but the main difference is a quantitative one (Greenbury *et al.*, 1963). While group $A_1$ cells have an average of 0.81 - 1.17 x $10^6$ group A antigen determinants per erythrocyte, group $A_2$ have an average of 0.24 - 0.29 x $10^6$ (Economidou *et al.*, 1967), although different results can be found (Cartron *et al.*, 1974) (refer to **Table 1.4**).

Some $A_2$ and $A_2B$ individuals can produce an antibody to $A_1$. Although not clinically significant unless of high titre and reactive at 37 °C, it is considered important to detect anti-$A_1$ in pre-transfusion testing of both donors and patients. If $A_2$ or $A_2B$ individuals with anti-$A_1$ in their serum were grouped only as group A or AB, their blood donation would be available for transfusion to group $A_1$ or $A_1B$ individuals. The anti-$A_1$ could react with the $A_1$ cells and cause a transfusion reaction, but only if

reactive at body temperature. It is important to detect specific weak subgroups in blood group testing. For example, the subgroup $A_x$ should be detected in blood donors as otherwise it may be grouped as an O. Current guidelines state that this subgroup must be detected (Guidelines for the Blood Transfusion Services in the United Kingdom, U.K. Blood Transfusion Services, 2002), yet the subject has been much debated. The risk caused by misgrouping an $A_x$ as a group O is theoretical and there are few cases involving transfusion reactions caused by transfusion of such blood to a group O (review, Issitt and Anstee, 1998).

**Table 1.3** Expected reaction pattern of the main ABO blood types, where + is a positive reaction and – denotes a negative reaction.

| | $A_1$ blood | $A_2$ blood | B blood | O blood | AB blood |
|---|---|---|---|---|---|
| Anti-B | - | - | + | - | + |
| Anti-A | + | + | - | - | + |
| Anti-A,B | + | + | + | - | + |
| $A_1$ cells | - | - | + | + | - |
| B cells | + | + | - | + | - |
| O cells | - | - | - | - | - |

Routine blood testing consists of testing erythrocytes with anti-A, anti-B and anti-A,B to determine presence or absence of the A and B antigens, and testing plasma/serum with known group $A_1$, B and O erythrocytes for the presence of ABO antibodies (Guidelines for the Blood Transfusion Services in the United Kingdom, U.K. Blood Transfusion Services, 2002). Testing of erythrocytes is known as forward grouping, and testing of plasma/serum known as reverse grouping. Although reverse grouping is classified as part of routine ABO grouping, it is determining the

presence or absence of antibody and therefore in this work will be considered as antibody screening for anti-A and anti-B and described as ABO reverse grouping. **Table 1.3** details the expected reaction patterns of the main ABO blood groups.

Circumstances exist where the reaction patterns in **Table 1.3** are weak or do not occur, such as in neonates, elderly people or individuals undergoing immunosuppressant therapy; these are cases where the immune system is not fully developed, in decline or suppressed.

### 1.2.1.2 The Rhesus Blood Group System

In 1939, Levine and Stetson discovered a case of haemolytic disease of the newborn caused by an antibody, which was later described as anti-D. In 1940, Landsteiner and Weiner made an antibody by injecting rhesus monkey erythrocytes into rabbits. The antibody agglutinated red cells from 85 % of samples, which were later described as RhD positive. There are now over 45 antigens in the Rhesus (Rh) system of which D, C, c, E and e are the most clinically significant (this system is reviewed in Daniels, 2002). RhD is highly immunogenic (Pollack *et al.*, 1971) and is therefore the most important Rhesus antigen in blood grouping. Rhesus antibodies tend to be IgG in class and generally do not activate complement.

The antigens of the Rh system are encoded by variant forms of the highly homologous genes *RHD* and *RHCE* located on chromosome 1 (see e.g. Daniels, 2002) and this theory was first forecast by Tippett (1986). The *RHD* gene encodes the RhD antigen and *RHCE* encodes the Cc and Ee antigens: two pairs of antithetical

antigens. Each gene encodes a polypeptide 417 amino acids in length which spans the red blood cell lipid bilayer 12 times, and functions as an ammonia transporter (Flegel and Wagner, 2002). 32 to 35 amino acid substitutions constitute the variations in the Rh phenotypes.

The RhD antigen has no antithetical counterpart, but consists of a mosaic like structure with multiple epitopes (Avent *et al.*, 2000). Individuals may lack one or more of these epitopes and produce an antibody to the part that they lack. Cases where RhD positive individuals have produced anti-D were investigated and in 1962, Tippett and Sanger reported six serologically different types of sample and described them as category I to VI. These individuals are now classed as 'Partial RhD', of which there are currently more than 20 types with DVI being the most clinically significant (Flegel and Wagner, 2002). The DVI antigen has very few of the RhD epitopes and is highly immunogenic. The DVI phenotype results from different homologous exchanges of exons from *RHCE* into *RHD*. In the U.K., donor testing reagents must detect DVI; and an antibody that detects this type must be included in donor testing. Partial RhD types can cause production of anti-D in RhD negative individuals, and have been implicated in haemolytic disease of the newborn (Mayne *et al.*, 1990). Also of concern in blood grouping are individuals classed as 'weak D'. These people have a complete RhD antigen but have fewer copies on the surface of the red cell, which can make detection more difficult. Weak D result from missense mutations in the RHD allele and are located in the transmembrane or intracellular segments of the protein (Flegel and Wagner, 2002). If weak D was undetected in donor blood and, therefore, classified as RhD negative, it could theoretically

stimulate anti-D production if given to RhD negative patients. Further details of the Rhesus antigens can be found in **Table 1.4.**

#### 1.2.1.3 The Kell Blood Group System

There are 23 antigens in the Kell blood group system (Schenkel-Brunner, 2000), the most clinically significant antigen being K. The K antigen is clinically significant as it is highly immunogenic, second only to RhD in terms of immunogenicity (Schenkel-Brunner, 2000). The K antigen is a single-pass transmembrane glycoprotein that is highly folded via disulphide bonds (Reid and Lomas-Francis, 1997), and considered to be a conformation-dependant antigen as it can be inactivated reaction with sulphydryl reagents (review, Schenkel-Brunner, 2000). Further details of the K and k antigens can be found in **Table 1.4.**

#### 1.2.1.4 The Duffy Blood Group System

There are six antigens in the Duffy blood group system (review, Schenkel-Brunner, 2000), $Fy^a$ being the most clinically significant and fairly immunogenic. The Duffy glycoprotein is a multi-pass transmembrane structure (Reid and Lomas-Francis, 1997). Further details of the $Fy^a$ and $Fy^b$ antigens can be found in **Table 1.4**. Other clinically significant blood group systems are described in **Table 1.4.**

### 1.2.2 Irregular Blood Group Alloantibodies

Testing for the presence of blood group alloantibodies in human plasma samples is mandatory in most countries worldwide. In the U.K. it is covered by the Guidelines for compatibility procedures in blood transfusion laboratories (Working Party of the

British Committee for Standards in Haematology Blood Transfusion Task Force, 2004) for antenatal and pre-transfusion testing and the Guidelines for the Transfusion Services in the United Kingdom (U.K. Blood Transfusion Services, 2002).

As such, the inclusion of this testing is critical in a new blood testing system. Irregular anti-erythrocytic antibodies are those produced to blood group antigens other than A or B, which do not occur naturally but are induced by immunisation with blood group antigen. An individual may produce an irregular erythrocytic antibody when they come into contact with blood that expresses an antigen 'foreign' to their own. This usually occurs either during pregnancy or following blood transfusion. Antibodies of this kind are usually IgG and for sensitivity require the use of an anti-human IgG as a secondary reagent in order to visualise an agglutination reaction. Detection of irregular antibodies in antenatal testing is important to prevent haemolytic disease of the foetus and newborn.

Regulations for irregular blood group alloantibody screening vary depending on whether testing donor or patient samples. The presence of such antibodies in a patient sample is of far greater clinical significance than if detected in a donor. This is because donor erythrocyte components have most of the plasma, and therefore antibody, removed and any residue is subject to dilution on transfusion. However, if antigen positive blood is introduced to an antibody producing patient it can lead to a severe transfusion reaction.

In the U.K. the technique used for irregular blood group antibody screening must be capable of detecting anti-D at 0.5 International Unit/ml or lower in donors, and 0.1 International Units/ml in patients (Guidelines for the Blood Transfusion Services in the United Kingdom, U.K. Blood Transfusion Services, 2002). There are no limits for other antibody specificities. Samples found to be positive in an antibody screen must be further tested for specificity.

Currently, erythrocytes from human blood are used in antibody screening tests and as blood typing controls, which means reliance on the constant despatch/receipt/change over of short shelf life products. The use of alternative source antigens or more stable erythrocyte antigens would both save precious donor blood as well as give a more consistent, reproducible and stable product. In Chapter 6, a solid-phase microarray method using various antigen-presenting formats will be explored for use as an antibody screening method.

### 1.2.3 Blood Borne Pathogen Testing

Mandatory blood donation testing includes testing samples for the presence of Hepatitis B Surface Antigen (HBsAg), Human Immunodeficiency Virus 1 and 2 antibodies, Hepatitis C Virus antibodies and Syphilis antibodies. Microbiology testing was not a subject of this project, but is considered later for potential inclusion in a blood testing microarray.

**Table 1.4a.** Blood group antigens: structure and attachment to the erythrocyte; the size and number of antigenic determinants per cell (if positive for that antigen); and frequency (in Caucasian Europeans). Information collated from Schenkel-Brunner (2000), Daniels (2002), and Reid and Lomas-Francis (1997). Continued on next page.

| Antigen | Blood Group System | Antigenic Determinant/Protein Structure | Attachment to Erythrocyte | Size | Antigen Number Per Erythrocyte | Frequency of Antigen |
|---|---|---|---|---|---|---|
| H (group O) | ABO | Fucose determinant attached to oligosaccharide chain | Carried on glycosphingolipid or glycoprotein. Expressed on N-glycans carried on band 3, 4.5, RH glycoprotein and CHIP | Branched structures, >200 kDa | 1.5-2 million | (H antigen frequency~100%) Group O = 46.7 % |
| A | ABO | N-Acetylgalactosamine determinant (GalNAc) attached to H precursor | Attached to H antigen (see above) | Branched structures, >200 kDa | A1 – 810K-1.170million<br>A2 – 160-440K<br>Ax – 1.4-10K | A antigen – 40%<br>A1 antigen – 34% |
| B | ABO | Galactose determinant (Gal) attached to H precursor | Attached to H antigen (see above) | Branched structures, >200 kDa | 610-830K | 11% |
| D | Rhesus | Protein/lipid aggregates. | Multi-pass membrane protein (involved in ion transport). There are 8 substitutions on extracellular loops. | Rh protein 30-32kDa Protein 417 amino acids in length | Dependant on phenotype:<br>Common:10-30K<br>Weak D:200-10K<br>High: 75-200K | 85 % |
| C | Rhesus | Protein/lipid aggregates. | Isoleucine position 60, Serine position 68, Serine position 103, of RhCE polypeptide. Cysteine residue at position 16. | Rh protein 30-32kDa Protein 417 amino acids in length | ~40K | 68% |
| c | Rhesus | Protein/lipid aggregates. | Leucine position 60 Asparagine position 68 Proline at position 103 of RhCE polypeptide. Cysteine/tryptophane at position 16 | Rh protein 30-32kDa Protein 417 amino acids in length | ~40K | 80% |
| E | Rhesus | Protein/lipid aggregates. | Proline at position 226 ($4^{th}$ extracellular loop) of RhCE polypeptide | Rh protein 30-32kDa Protein 417 amino acids in length | ~20K | 29% |
| e | Rhesus | Protein/lipid aggregates. | Alanine at position 226 ($4^{th}$ extracellular loop) of RhCE polypeptide | Rh protein 30-32kDa Protein 417 amino acids in length | ~20K | 98% |
| $C^w$ | Rhesus | Protein/lipid aggregates. | Glutamine at position 41 of RhCE polypeptide | Rh protein 30-32kDa Protein 417 amino acids in length | $2.15 – 4 \times 10^4$ | 2% |

**Table 1.4b.** Blood group antigens: structure and attachment to the erythrocyte; the size and number of antigenic determinants per cell (if positive for that antigen); and frequency (in Caucasian Europeans). Information collated from Schenkel-Brunner (2000), Daniels (2002), and Reid and Lomas-Francis (1997). Continued on next page.

| Antigen | Blood Group System | Antigenic Determinant/Protein Structure | Attachment to Erythrocyte | Size | Antigen Number Per Erythrocyte | Frequency of Antigen |
|---|---|---|---|---|---|---|
| K | Kell | Methionine at position 193 | Single-pass membrane protein. Glycoprotein, N-glycan residues at sites 93,115,191345,627 | Protein 732 amino acids in length (93kDa glycoprotein) | 3.5-18K | 9% |
| k | Kell | Threonine at position 193 | Single-pass membrane protein. Glycoprotein, N-glycan residues at sites 93,115,191345,627 | Protein 732 amino acids in length (93kDa glycoprotein) | 3.5-18K | 99.8% |
| $Fy^a$ | Duffy | Glycoprotein, N-glycans attached at 2 sites.<br>Glycine at position 42 | Multi-pass membrane glycoprotein | Protein 336 amino acids in length (35-43kDa) | 13.5K | 66% |
| $Fy^b$ | Duffy | Glycoprotein, N-glycans attached at 2 sites.<br>Aspartic acid at position 42.<br>Residues 38,39,50,55 and 58 essential for specificity. | Multi-pass membrane glycoprotein | Protein 336 amino acids in length (35-43kDa) | 13.5K | 55.7% |
| $Jk^a$ | Kidd | One N-glycosylation site on extracellular side at Asparagine at position 211).<br>Asparagine at position 280 | Multi-pass membrane protein | Protein 391 amino acids in length (43kDa) | 14K | 77 % |
| $Jk^b$ | Kidd | Asn at position 280 | Multi-pass membrane protein | Protein 391 amino acids in length (43kDa) | 14K | 74 % |
| S | MNS | GPB Amino acid dimorphism at position 29: methionine | Single pass membrane protein GPB | GPB=72aa (24kDa) | GPB per erythrocyte=200K | 55% |
| s | MNS | GPB Amino acid dimorphism at position 29: threonine | Single pass membrane protein GPB | GPB=72aa (24kDa) | GPB per erythrocyte=200K | 89% |

**Table 1.4c.** Blood group antigens: structure and attachment to the erythrocyte; the size and number of antigenic determinants per cell (if positive for that antigen); and frequency (in Caucasian Europeans). Information collated from Schenkel-Brunner (2000), Daniels (2002), and Reid and Lomas-Francis (1997).

| Antigen | Blood Group System | Antigenic Determinant/Protein Structure | Attachment to Erythrocyte | Size | Antigen Number Per Erythrocyte | Frequency of Antigen |
|---|---|---|---|---|---|---|
| M | MNS | GPA Amino acid dimorphism in N-terminal segment:<br>Serine position 1, glycine position 5.<br>Anti-M detection relies on presence of O-glycans at aa residues 2,3&4. | Single pass membrane glycoprotein | GPA=131 aa (37kDa), | GPA per erythrocyte=800K | 78% |
| N | MNS | GPA (& GPB) Amino acid dimorphism in N-terminal segment:<br>Leucine position 1, glutamic acid position 5.<br>Anti-N detection relies on presence of O-glycans at aa residues 2,3&4. | Single pass membrane glycoprotein | GPA=131 aa (37kDa), | GPA per erythrocyte=800K | 72% |
| P1 | P | Addition of galactosyl residue to paragloboside | Linked to glycosphingolipid through ceramide.<br>Paragloboside precursor.<br>Terminal $\alpha$-Galactose determinants | Variable | Up to 500K | 79% |
| $Le^a$ | Lewis | Fucose added to precursor subterminal N-acetyl-D-glucosamine | Located on glycosphingolipids adsorbed onto rbc from plasma | Branched structures, >200 kDa | Variable – attached by passive adsorption. | 22% |
| $Le^b$ | Lewis | 2 Fucose added to precursor terminal galactose and N-acetyl-D-galactosamine | Located on glycosphingolipids adsorbed onto rbc from plasma | Branched structures, >200 kDa | Variable – attached by passive adsorption. | 72% |

## 1.3 Specifics of Blood Testing

### 1.3.1 Mandatory Blood Testing

The purpose of blood donation testing was discussed at the start of this chapter. The majority of current blood donation testing takes the form of either haemagglutination assays or enzyme-linked immunosorbant assays (ELISA). The tests described in **Table 1.5** are mandatory in the UK and must be performed on every blood donation (Guidelines for the Blood Transfusion Services in the United Kingdom, U.K. Blood Transfusion Services, 2002). Additional testing may be performed on selected donations for specific purposes, i.e. testing for antibodies to cytomegalovirus or malaria, human leucocyte antigen typing, extended blood typing for C, E, C, e and K antigens. In Scotland, mandatory testing currently also includes nucleic acid amplification techniques (NAT) for Human Immunodeficiency Virus. Patient testing is somewhat different and routinely involves only blood typing and blood group alloantibody screening assays.

### 1.3.2 Techniques Used in Blood Testing

#### 1.3.2.1 Haemagglutination Assays

In blood group serology the interaction of antibody (Ab) on the erythrocytes (red blood cells) with the corresponding antigen (Ag) is routinely detected by haemagglutination assay. The technique of haemagglutination was discovered in 1888 by Stillmark (reviewed in Plapp *et al.*, 1984), who observed that extracts of *Ricinus communis* seeds strongly agglutinated erythrocytes. There are two stages to a haemagglutination reaction: sensitisation and agglutination. Sensitisation involves binding of antibody to antigen and is influenced by several factors.

**Table 1.5.** Mandatory blood donation testing in the United Kingdom, as of September 2005 (Guidelines for the Blood Transfusion Services in the United Kingdom, U.K. Blood Transfusion Services, 2002).

| TEST | PURPOSE | FORMAT |
|---|---|---|
| ABO Blood Typing | Typing of donor red blood cells for blood group antigens of the ABO blood group system | Haemagglutination |
| ABO Reverse Typing (first time donors only) | Typing of donor plasma for blood group antibodies of the ABO blood group system | Haemagglutination |
| Rhesus D Typing | Typing of donor red blood cells for the presence/absence of the Rhesus D blood group antigen | Haemagglutination |
| Erythrocytic Alloantibody Screen | Testing of donor plasma for the presence of irregular antibodies to blood group antigens | Haemagglutination |
| Hepatitis B Surface Antigen (HBsAg) | Testing of donor plasma for the presence of Hepatitis B Surface Antigen | ELISA |
| Human Immunodeficiency Virus Types 1 and 2 (HIV 1 & 2) | Testing of donor plasma for the presence of HIV 1 & 2 antibodies | ELISA |
| Hepatitis C Virus | Testing of donor plasma for the presence of HCV antibodies and RNA | ELISA/HCV-NAT |
| Syphilis | Testing of donor plasma for the presence of syphilis antibodies | Agglutination |

Agglutination may or may not happen after sensitisation, and is dependant on the type of antibody involved – an IgM molecule can cause direct agglutination while an IgG molecule usually requires a secondary antibody to cause indirect agglutination. Haemagglutination reactions are reversible, and can therefore be broken up and potentially lead to false negative reactions. Excessive agitation, whether by manual

or automated means, can give rise to this. Voak (1999b) explained that the major cause of false negative reactions in indirect haemagglutination assays was in fact attributed to excessive agitation.

IgG molecules have two antigen-binding sites approximately 140 Å apart, whereas an IgM molecule has ten antigen-binding sites approximately 300 Å apart (review, Issitt and Anstee, 1998). IgM antibodies tend to agglutinate red cells in a one-stage test due to their size and number of antigen-binding sites; the molecule links between erythrocytes more easily than an IgG molecule and cross-links, forming agglutinates.

The number and location of Ag sites can facilitate binding of Ab. For example, antigens of the ABO blood group system protrude from the erythrocyte membrane and are present in large numbers, whereas those of the Rh system are closer to the membrane and are present in lower numbers. Therefore, ABO sites are more readily accessible than Rhesus Ag sites.

The concentration of Ag-Ab involved in the interaction can vary the sensitivity of the test. If the amount of Ab present is increased the Ab:Ag ratio increases and the stronger the observed reaction. If the concentration of Ag in a test is increased or decreased this too will influence the amount of Ab bound to each antigen. pH can affect the conformation of the protein molecule as well as its function, which is often pH dependent. Most antibodies are optimal between the pH value of 6.5 and 7.5 (Issitt and Anstee, 1998), although there are exceptions. Most monoclonal anti-M, for example, react optimally at pH 8.5.

The ionic strength of the test environment influences the rate at which Ag-Ab complexes form. Erythrocytes are negatively charged and, therefore, repel each other due to the ionisation of carboxyl groups of N-acetyl neuraminic acid residues, or sialic acid. When suspended in an ionic solution, free cations are attracted to the negative charge on the cell surface, and this forms a repelling cloud around the cell. This electron repulsion is known as the zeta potential (Pollack and Reckel, 1970; 1977). Adjusting the concentration of free cations in the environment alters the charge on the red cell, which alters the zeta potential. Reduction in zeta potential facilitates Ab agglutination as well as sensitisation. For example, when the ionic strength of the environment is decreased the zeta potential of red cells is increased and this leads to a greater attraction between the red cells and the usually oppositely charged Ab. The ionic strength of haemagglutination reactions is usually lowered by the use of low ionic strength saline (LISS) in which the red cells are suspended. Enzymes can also be used to lower cell repulsion by removing sialic acid and other proteins from the erythrocyte surface.

Temperature can greatly influence the rate of Ag-Ab reaction. Lower temperatures can increase binding avidity but reactions happen more quickly at warmer temperatures (such as 37 °C). Temperature can also affect the conformation of the antigen (Kelton *et al.*, 1984).

As many factors can influence haemagglutination reactions, it could be advantageous to omit the agglutination stage, and detect the sensitisation interaction in alternative ways. Solid-phase formats rely only on sensitisation of antibody and antigen, and not

agglutination, and this will be further discussed. Blood group serological testing involving sensitisation and haemagglutination can be performed by a number of different techniques as follows and varies depending on the requirement of the laboratory.

- **Slide testing**: This is the simplest form of test and tends to be the least sensitive. It involves mixing Ab with erythrocytes on a glass slide, and viewing over a light box to determine the presence or absence of haemagglutination. This test can be very subjective and is not automated, and depending on operator experience, throughput can be limited. Like all manual techniques, slide testing requires good training on technique and the use of controls, and data recording has to be performed manually.

- **Tube testing**: The Ab and erythrocytes are added to a glass tube and mixed. Centrifugation must follow Guidelines for compatibility procedures in blood transfusion laboratories (Working Party of the British Committee for Standards in Haematology Blood Transfusion Task Force, 2004) and usually follows an incubation period. Centrifugation pellets the cells at the bottom of the tube in a 'cell button'. Centrifugation makes the test more sensitive than the slide technique, but this is not automated and depending on operator experience, throughput can be limited and errors more likely due to human error.

- **Microplate Testing**: 96 well microplates are used in which the Ab and erythrocytes are mixed and incubated. The reagents are dispensed when required

by an automated system; the plates are not usually supplied with pre-dispensed reagents. This is usually automated and is, therefore, suited to high throughput testing as required in blood donation testing. Depending on the system used, centrifugation may or may not be used. Computer-driven spectrophotometry or digital photography are used to capture images and interpret the reaction.

- **Micro Column Technology** A gel card usually consists of six microwells each containing a 'gel' of either glass beads (Ortho BioVue system) or Sephadex (dextran derivative) (DiaMed ID System). The Ag-Ab reaction takes place above the gel during an incubation period, or the Ab can be suspended in the gel. The card is then centrifuged. A positive reaction, demonstrated by haemagglutination, cannot pass through the spaces in the gel and forms a line at the top of the gel, or partial movement through the gel. Non-agglutinated cells can easily pass through the spaces in the gel and the negative reaction demonstrated by erythrocytes at the bottom of the gel. The column technique was patented by DiaMed AG and described by Lapierre *et al.*, (1990). This system is now available with varying degrees of automation. Although suitable for high-throughput testing, it is more often used for patient testing in lower throughput hospital laboratories or blood banks rather than for donor testing. An advantage of the micro column systems is that there is a reduced risk of infection of staff as the system allows enclosed testing and each card is pierced prior to use, although the costs of this system can be limiting to some laboratories.

- **Solid Phase Antibody Screening:** This differs from simple microplate testing as erythrocyte membranes are immobilised on microplate wells and used for the detection of erythrocytic alloantibodies in plasma or serum. Existing systems use 'indicator' erythrocytes that demonstrate visible haemagglutination in positive tests. Currently, these systems (which can be automated) are only used for blood group alloantibody screening. Methods for solid-phase antibody screening will be discussed further in section 1.3.2.5. dealing with the technology available in solid-phase systems.

- **Flow cytometry:** This method does not rely on agglutination, only sensitisation. It has been used to demonstrate the presence or absence of Ag-Ab reactions using fluorescently labelled antibodies. Although sophisticated and highly sensitive, the flow cytometer method is complex and not suitable for high-throughput testing.

### 1.3.2.2 Enzyme-Linked Immunosorbant Assays (ELISA)

Protein based testing for the presence or absence of pathogenic microorganisms, or antibodies to them, usually takes the form of an ELISA, which are mostly performed in microplates. This allows the testing of many blood donations at any one time. The ELISA method can be adapted to perform blood group serological assays (Scott, 1991), but is not routinely used.

### 1.3.2.3 Nucleic Acid Amplification Testing (NAT) of Transfusion Related Antigens

Recently NAT has been introduced for pathogen testing of donations. This type of testing gives increased sensitivity over ELISAs. NAT uses the polymerase chain reaction (PCR) to amplify viral RNA or DNA present in pooled samples. Pooled samples that give a positive reaction are cross-pooled and tested to determine positive donations, although there are now some automated systems available that can perform NAT testing on individual samples.

While NAT techniques are used for pathogen testing of blood donations, it has not been fully adopted as a routine method for blood typing. Genes encoding most of the blood group systems have been cloned and sequenced and generally it has been found that blood group antigens arise from single-nucleotide polymorphisms (SNPs). The ABO and Rhesus systems pose more of a challenge; as the antigens do not arise from SNPs, their genetic basis is more complex and the current most reliable method of grouping remains agglutination (Anstee, 2005).

For human platelet antigens much work has been done to prove that typing can be done by large-scale NAT methods, (Bugert *et al.*, 2005) based on the use of SNP detection.

Denomme and van Oene (2005) attempted blood group genotyping, but with a level of error – 98 % of samples were correctly typed, but with less success for Rhesus groups. Beiboer *et al.* (2005) also reported on rapid blood group genotyping. Hashmi

*et al.* (2005) performed DNA typing using bead array technology from Illumina, the BeadChip™, discussed later, compared to manual PCR. In the bead array, SNPs were attached to the beads and then analysed after hybridisaton. They found comparable results by both methods and have developed a prototype chip, but the ABO and Rhesus systems were not tackled. The system seems more for screening purposes than an outright replacement for existing test procedures. Systems such as these may help with blood group antibody shortages to certain antigens, as only confirmatory testing is required. For purposes other than blood typing, Zhang *et al.* (1999) demonstrated that DNA genotyping could be performed directly from whole blood, without prior processing steps. This could potentially cut testing time if applied to blood group genotyping.

While performing DNA based testing of genotypes it is important to remember that sometimes the genotype and phenotype do not correlate (Reid, 2003). This may be due to a number of circumstances. Certain medical practices can lead to the presence of non-self DNA i.e. transfusion/transplantation. Molecular events such as a point mutation in a regulatory element of a gene could impair transcription, or a mutation in splicing sites, a premature stop codon, the absence of an interacting protein or less protein in the membrane of the erythrocyte could all cause discrepancies. In some cases a PCR primer may not be able to bind efficiently to a target, yet a product is present on the erythrocyte membrane. For example hybrid genes, when part of the *RHD* gene is replaced with the *RHCE* gene, primers for the *RHD* gene will not amplify yet a partial RhD will be present on the surface of the red cell (Avent and

Reid, 2000). Anstee (2005) also reports that molecular methods are 'as yet' not error-free, and that mutations will continue to evolve.

Although the technology of DNA based genotyping is unlikely to be employed for routine blood typing in the very near future, it has powerful potential for transfusion medicine through its accuracy and sensitivity (Reid and Rois, 1999; Petrik, 2001). Currently the two most valid uses for blood group genotyping are to determine the RhD type of foetuses of women with anti-D, and to predict phenotype of transfusion-dependent patients (Daniels *et al.*, 2005). It is considered very important to continue serological testing and this will be explored within this work by looking at the evolving technology of microarrays, in which Ab-Ag interactions are interpreted without the use of haemagglutination.

### 1.3.2.4 Automation in Blood Testing

Levels of automation in blood testing are constantly increasing as automation gives more security in positive sample identification and can reduce overall costs. Tests can be performed simultaneously, yet these are still carried out as individual tests on multiple instrument platforms due to the different formats described above. This means that results from multiple testing platforms have to be reconciled at a computer interface, often leading to delays. Many different pieces of equipment mean that more than one blood sample is required for testing. If multiple tests were performed on one platform such as microarray it would mean fewer samples of reduced volume would be required, which is of particular benefit to patients, and results could be available more quickly. A microarray system would enable

multiplexing and full automation. This would aid the provision of donor blood and the selection of compatible blood for patients. A table comparing different techniques and throughputs is available in the paper Robb *et al.* (accepted 2005 for Transfusion Medicine) in Appendix 4.

Currently most blood donation testing is carried out using the Olympus PK7200 or PK7300 systems, which carries out mandatory agglutination assays (blood typing and Syphilis). The cost and throughput of the Olympus system is too high for use in testing of patient samples (it can test 240 samples in one hour). It is also considered of lower sensitivity when testing for the presence of blood group alloantibodies, which is acceptable for the testing of donor blood. Using the Olympus system, the most commonly used high throughput method for blood group alloantibody screening of blood donations involves the use of group O erythrocytes that are papain treated (to detect IgG antibodies without the use of a secondary antibody). This testing uses a microplate format with stepped wells. The erythrocytes are prepared and dispensed into the microplate shortly before the addition of the donor serum. Incubation takes place and then the plate is read. Donation testing centres commonly use the Abbott Prism® (Wiesbaden, Germany) system for performing pathogen testing.

Column agglutination testing is routinely used in patient testing blood banks throughout much of the developed world for antibody screening. Considering the sensitivity, throughput and automation available in parallel with costs, these systems are considered optimal for patient testing and mostly for lower throughput antibody

screening. The two most popular systems in use in the U.K. are those supplied by DiaMed AG (ID System) and Ortho Clinical Diagnostics (BioVue), although others are now available. The column agglutination methods are often referred to as solid-phase methods in the field of blood grouping. However, this does not comply with the definition that solid-phase means the immobilisation of a component of the test. The antibodies used in column agglutination methods are simply mixed into the matrix, not immobilised. The automated reading of this system uses a charge-coupled device camera.

### 1.3.2.5 Solid Phase Methods for Use in Blood Testing

There are existing techniques that use solid phase techniques rather than the traditional liquid phase methods. For this, solid phase refers to the immobilisation of the antigen or antibody to a surface before interaction with analyte/target solution. Advantages of solid-phase methods can include the pre-dispensing of reagents, which saves greatly on testing time, and the possibility of increased automation and reading. As microarrays are solid-phase platforms and will be used in this work, previous development work performed in microplates is very relevant.

The immobilisation of antibodies to microplates for the purpose of blood typing is perhaps a more simple approach than that of antigen immobilisation for antibody screening. Fewer publications are available on microplate methods for antibody screening as the requirement for washing and the addition of a secondary detection antibody makes the microplate process more complicated. Complications also arise due to the complex nature of the antigens required for use in antibody screening,

which are immobilised on the erythrocyte, and can therefore also be considered in solid-phase.

Investigations into non-haemagglutination based assays began in the 1950's. The 96-well format for microplates was first used in viral serological investigations, then in 1966 it was described in serological tests for blood group serology (reviewed in Plapp *et al.*, 1984). During the 1960s the idea of anti-globulin coated indicator cells was reported by Fagraeus and Espmark, and in the 1970s many groups were reporting variations of solid-phase assays involving erythrocytes and platelets in solid-phase (reviewed in Plapp *et al.*, 1984). Solid-phase methods for blood typing and alloantibody screening are discussed below.

#### 1.3.5.2.1 Solid Phase Methods for Blood Typing

To perform blood typing in solid-phase, blood typing antibodies must be immobilised onto a surface. This has been already been demonstrated in plastic microplates and, compared to antibody screening, is considered relatively simple.

In 1985, Sinor *et al.* described a method for solid-phase ABO and RhD typing, and they also reported work on solid-phase antibody screening methods. They encountered problems with stability of both immobilised antibodies for ABO and RhD grouping and immobilised erythrocytes for reverse ABO grouping and antibody screening. For ABO and RhD grouping, Sinor *et al.* overcame problems by using immobilised $A_1$ RhD negative, B RhD negative and O RhD positive erythrocytes onto microplates. Once immobilised the cells were then sensitised with IgM

antibodies against blood group antigens A, B and RhD. The ABO and RhD type was determined when the patient/donor erythrocytes were added and bind to the appropriate immobilised antibodies, and a positive reaction was demonstrated by a layer of erythrocytes over the well. The same group (Beck *et al.*, 1985) also reported the use of salivary substances to bind blood typing reagents to perform ABO grouping.

A dipstick solid-phase method has been developed for use in bedside transfusion testing (Knight and de Silva, 1996). This consists of a stick onto which IgM blood typing antibodies were bound. This stick is dipped into a blood sample and then rinsed with saline. The results are visualised with the presence or absence of the red colour, and therefore haemagglutination, of bound erythrocytes. One disadvantage to this system would be the lack of ABO reverse typing and due to this its use is limited.

Scott (1991) reported on the development of a solid-phase microplate system for blood typing. A direct correlation between the amount of antibody immobilised and the level of binding of erythrocytes to immobilised antibody was evident, yet too high a concentration resulted in less coating, which could possibly be attributed to the 'hook effect' (Fernando and Wilson, 1992). Critical factors were shown to be the type of microplate used, purity of antibody, concentration, pH and ionic strength of coating buffers.

Uthemann *et al.* (1999) developed a solid-phase method for ABO, Rhesus and K grouping using immobilised monoclonal IgM antibodies for forward grouping and erythrocyte membranes for reverse grouping. The main difference to previously described methods was due to the realisation that passive adsorption of the antibody occurred even if added immediately before the cells, therefore allowing erythrocyte adherence over the well. Other variations of blood typing in solid-phase are numerous.

Diagast (Loos Cedex, France, Diagast.com) have developed a solid-phase blood typing system that does not rely on centrifugation. In this system they describe plates pre-coated with antibody to which test cells are added. These cells are diluted in a magnetising solution. Rather than centrifugation, the plates are held over a magnetic source, which pellets the magnetised cells. After this the plate is agitated and agglutination demonstrated by a pellet, whereas cells in solution demonstrate a negative reaction. To complement this system they have an antibody screen system where the reagent cells are pre-magnetised.

In 2003, Hurt *et al.* reported on a compact disc system for ABO and RhD typing. They claimed that the system also had potential for pathogen testing, but no commercial progress has been reported.

#### 1.3.2.5.2 Solid Phase Methods for Alloantibody Screening

The basic principles of solid-phase antibody screening are simple; a surface must be coated with blood group antigen, sample serum/plasma is added, unbound

serum/plasma proteins are washed away, antibody may or may not be detected to give a positive or negative result. As in ELISAs, the technique is more complicated in practice. Considerations include the optimal conditions/substrate for immobilisation of antigen, the format of the antigen itself, the blocking procedures and reduction of non-specific binding, incubation times and the detection antibody properties. Investigated methods for blood group antibody screening have included the immobilisation of the whole red cell. Methods based on other antigen types or cells have included the immobilisation of cell membranes, cell fragments, peptides, synthetic antigens and aptamers. These may be potential options if applied to blood testing microarrays.

In 1985, Beck *et al.* reported the use of salivary ABO blood group substances in a microplate method for ABO grouping. Later they reported that these substances could be used for reverse grouping to detect anti-A and anti-B. Beck *et al.* found that the salivary substances bound readily to the plastic microplate.

In 1986, a semi solid-phase microplate indirect antiglobulin test was reported (Ross and Gordon, 1986; review, Scott, 1991), and this technique was used routinely in some laboratories. However, this was not a total solid-phase method as only the incubation with the immobilised secondary antibody (anti-human globulin reagent) took place in the microplate environment. Incubation of serum and erythrocytes was performed by standard liquid phase techniques. To develop a totally solid-phase antibody screening method it is clear that the blood group antigen must be immobilised on a surface. Failure to do this means the test system would rely on

transfer from one reaction chamber to another and involve more time and manipulations.

Erythrocytes or erythrocyte membranes do not bind to microplates as their surface is negatively charged, as is the microplate surface. This can be overcome by treatment of the surface to enhance binding conditions. Scott (1991) used poly-L-lysine-coated microplates, antibodies to erythrocytes and the use of castor bean lectin (a non-specific binder of all erythrocytes). The poly-L-lysine coated plates were positively charged and subsequently bound erythrocytes readily. Peroxidase substrate was added to lyse the cells before measuring the colour change in the well, and this indicated the level of erythrocyte binding. Poly-L-lysine gave the best results. These are used often in DNA microarrays and will be explored in this thesis for use in blood testing.

Stocker and Heusser (1979) reported that cells (erythrocytes and lymphoid cells) could be bound to a microplate by the use of gluteraldehyde fixation or by antibodies bound to the plate. The resulting cell layers were used in a radio-immunoassay to screen monoclonal antibodies. Although the cell layers were stable for short periods, there were doubts over the stability of erythrocyte antigens following this treatment (Scott, 1991).

Scott (1991) and Sinor (1992) found that erythrocyte ghosts were more stable in solid-phase than whole cells, and this fact may be used in the development of a microarray-based system. For quality control of bound ghosts, Scott used a positive

control antibody to determine the level of binding to the plate. The antibody screening system worked well when testing purified monoclonal antibodies. However, when human serum/plasma samples were tested, high non-specific binding was evident. For the testing described, an enzyme-linked antiglobulin reagent was used for detection. It appeared that high levels of immunoglobulin from the serum were binding to the immobilised ghosts. Blocking with casein, albumin or Tween did not alleviate the problem. Other groups were also working on the development of solid-phase antibody screening systems and finding similar problems. As described below, the problem was overcome using 'indicator cells'.

A group, which later became associated with the company Immucor (Norcross, GA, USA), began the development of solid-phase red cell adherence assays for routine blood transfusion testing during the 1980s. Sinor (1992) described the four main problems they encountered. Firstly they sought a method for immobilisation which did not rely on binding of erythrocytes to immobilised antibody, as they felt this would be unreliable due to problems in automated wash systems. They overcame this by using chemical coupling agents for immobilisation of erythrocytes, followed by the lysis of the erythrocytes in-situ to create membranes. The detail of the attachment chemistry was not published. To perform ABO reverse grouping and antibody screening, whole erythrocytes were immobilised and dried. Patient or donor serum/plasma would then be added. If antibodies were present they bound onto the immobilised erythrocytes. Binding of antibody was demonstrated by the addition of 'indicator red cells'; erythrocytes coated with anti-IgG. These will bind to either anti-A or anti-B which has bound to the immobilised A or B erythrocytes, or to the

alloantibodies from serum/plasma bound to the antibody screening immobilised erythrocytes. The indicator cells are prepared by sensitising the erythrocytes with an IgG antibody, and then incubation with anti-IgG. The preparation process ensures this step does not result in haemagglutination.

The final problem described by Sinor (1992) was that of non-specific binding of IgG in human serum/plasma samples, which can be common to microplate based assays. They discovered that this could be solved by centrifugation of the microplate at two different speeds, one low, and one high. The low centrifugation allows the indicator erythrocytes to bind specifically, while the higher centrifugation pellets the erythrocytes in a negative test without reducing the adherence of the indicator erythrocytes in a positive test.

Immucor introduced this technology as the Capture R™ antibody screening system in 1989. This system is a total solid-phase system, i.e. all the testing is performed in one microplate well. Originally this system was supplied with activated microplates to which the user had to add reagent erythrocytes to immobilise them. Latterly the system was enhanced by the use of plates pre-coated with erythrocyte ghosts. Immucor also released similar technology for the testing of Cytomegalovirus and Lyme Disease antibodies. The Food & Drug Administration (FDA) in the United States of America, perhaps the most stringent regulatory system, has approved the Immucor testing systems. However, a limitation of this system is evident. Rolih *et al.* (1995) and Zeiler *et al.* (1996) both report on solid-phase systems, and when using IgG coated indicator cells, failed to detect IgM alloantibodies.

There have been other reports on the use of membranes to create solid-phase alloantibody screening methods (Million *et al.*, 1998), which can also use different methods of detection such as antibody-coated beads. At this time, none are commercially available.

Biotest (Dreieich, Germany) released an antibody screening system in the early nineties. The Biotest SolidScreen is now a commonly used technique throughout Europe. The microplates in this system are coated with an antibodies to anti-human globulin reagent. To this, reagent erythrocytes, test serum/plasma and antiglobulin reagent are added. If the cells are sensitised with antibody they will bind the anti-globulin reagent and then attach to the microplate.

#### 1.3.2.5.3 Biacore – A Solid Phase Platform for the Study of Biological Interactions

One of the most sophisticated methods to study biological interactions in solid-phase is the Biacore platform using the detection method of surface plasmon resonance (SPR). SPR is a well-recognised method for the study of immobilised proteins, peptides, nucleic acids and other molecules for analysis. SPR systems are considered solid-phase and can be used to monitor real-time biological interactions without associated labelling of the samples (Yuk and Ha, 2005). The use of unlabelled biomolecules is advantageous as the sample preparation steps are reduced. Also, the labelling of some molecules can affect their function (Fägerstam *et al.*, 1990). SPR is useful to provide more detailed analysis of interactions and specificities, and can be used for kinetic studies, but it is not a typical high throughput method. Currently, available SPR chips allow the study of one probe-ligand interaction at a time and is,

therefore, limited in its use and cannot be used in multiplex assays. However, there are groups working on the use of SPR in high-throughput systems (Yuk and Ha, 2005) in combination with other technologies.

SPR arises at the surface of a metal film when light is reflected onto it. Surface plasmons are free electrons on the surface of a film and are excited under specific conditions, therefore transferring energy. When energy transfer between the light and the metal match, this is known as resonance, and is seen as a dip in the reflected light intensity (Liebermann and Knoll, 2000).

SPR relies on total internal reflection (TIR): when a light beam from a medium of high refractive index meets an interface of a lower refractive index, the light is reflected at the interface and propagates back into the higher refractive index medium. Although the light beam is reflected back into the higher refractive index medium, it does leak an electrical field into the lower refractive index medium, and this is known as an evanescent wave, which extends 100 nm above and below the metal surface. In SPR systems the interface is coated with a conducting metal, which is in contact with the lower refractive index medium. The presence of the metal enhances the evanescent wave effect due to its being penetrated by and propagating the wave.

Any change in the electrical field results in a change in the wavelength of light that is absorbed rather than reflected. The magnitude of the change is quantitatively related to the magnitude of the chemical change, and therefore the bound mass. When

molecules bind to the sensor surface the mass increases, when they dissociate or are removed, it decreases. The amount of light reflected is measured by a reflectance spectrophotometer and the SPR angle is monitored by a change in the detector position (Fägerstam *et al.*, 1990), demonstrated in **Figure 1.6**. SPR detects changes in mass in the aqueous layer close to the surface by measuring the change in refractive index (RI), which is the degree to which the light is bent. The actual response is measured in response units (RU) and is proportional to the mass on the sensor surface (one RU represents a change of 0.0001 in the angle of reflective light (Biacore.com). SPR systems output data as sensorgrams, which are computer-generated plots of response against time showing the interaction progress.

SPR systems can be used to monitor interactions between molecules; one attached to the sensor surface and one in solution. However, the sensor chip detection method cannot differentiate between the binding of specific analyte and non-specific binding caused by binding of other molecules from the analyte solution (Brynda *et al.*, 2002).

The Biacore-X™ SPR system is used with Biacore Sensor Chip™ CM5 chips. In these chips a thin layer of gold (approximately 50 nm thick) covers the glass. **Figure 1.6** shows where the interaction takes place on the gold covered side of the sensor chip surface located on the opposite side from where light is reflected.

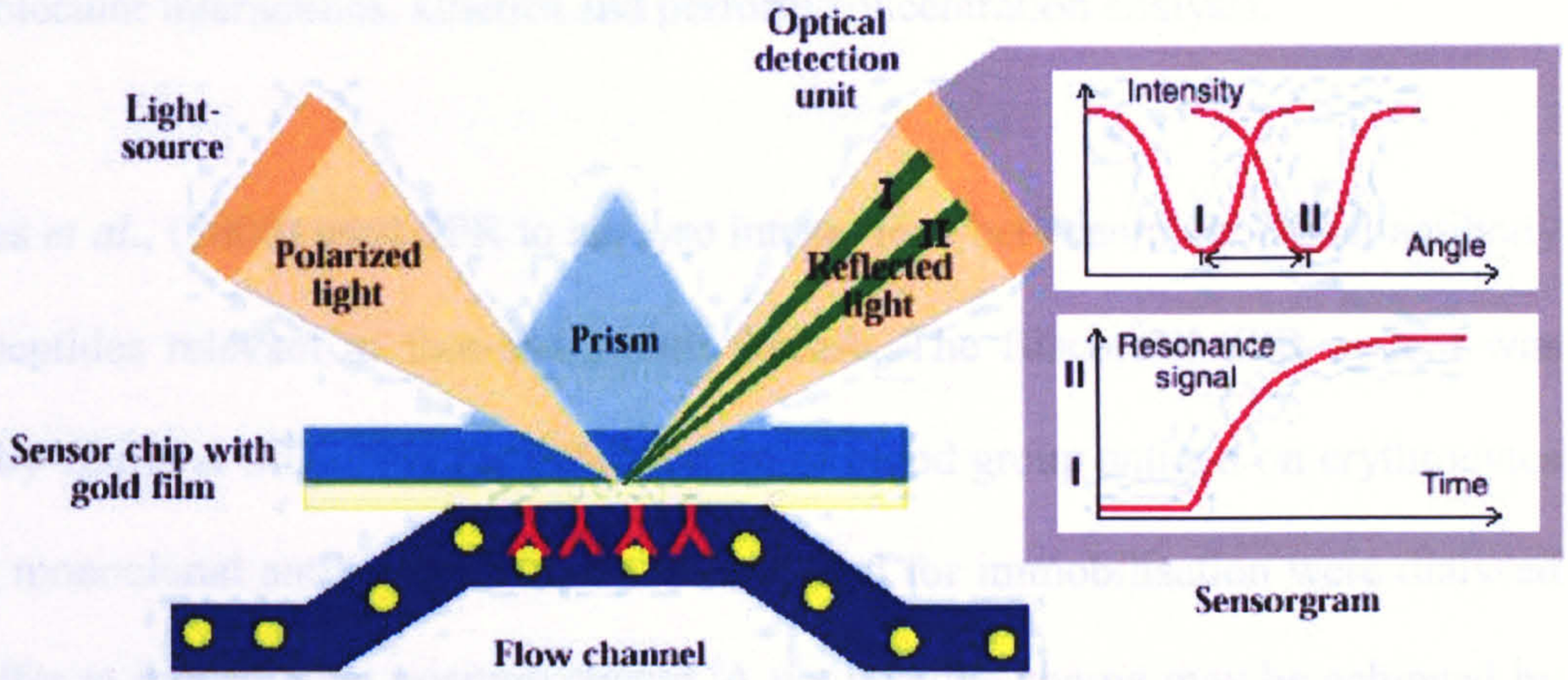



**Figure 1.6.** Diagram of an SPR flow cell (Biacore.com). The interaction takes place on the gold surface in contact with the flow cell. The analyte solution is passed through this flow cell under the control of a microfluidic system. The graphs show the effect of a change in the reflected light.

The analyte solution is passed through the flow cell in a precisely controlled microfluidic system that allows it to interact with the ligand. The Biacore Sensor Chip™ CM5 gold layer is modified by the attachment of a hydrophobic alkanethiol layer. A layer of flexible unbranched carbohydrate polymer named carboxymethylated dextran covers the alkanethiol.

This layer is hydrophilic, making it suitable for attachment of most biomolecules. At pH above 3.5, the 100 nm thick layer has many negatively charged carboxyl groups that are used for the covalent immobilisation of positively charged molecules. The flexible nature of the dextran allows free movement of attached ligands, and means they can be retained in a functional state and accessible in three dimensions. The Biacore Sensor Chip™ CM5 range can be used to covalently immobilise ligands

through -$NH_2$, -SH, -CHO, -OH or -COOH groups, and can be used to study biomolecular interactions, kinetics and perform concentration analysis.

Gomes *et al.*, (2000) used SPR to analyse interactions between monoclonal antibody and peptides relevant to foot-and-mouth disease. The Biacore™ SPR system was used by Quinn *et al.* (1997) for the detection of blood group antigen on erythrocytes using monoclonal antibodies. The antibodies used for immobilisation were dialysed in buffer to induce a net positive charge. A net positive charge may be achieved by lowering the pH of the Ab solution below its isoelectric point, which for IgM antibodies should be about pH 5.5 (Quinn *et al.*, 1997). The positive charge allows the antibody to be covalently immobilised onto the negatively charged surface.

The work performed by Quinn *et al.* (1997) was novel as it involved the detection of cells. The system demonstrated the specific reactivity of erythrocytes when passed over the sensor surface using SPR for the detection of the erythrocyte mass. Due to the size of an erythrocyte (7.8-8.3 $\mu$m in diameter) the bulk of the bound erythrocyte may be situated outside the evanescent field of the chip and they may also be unable to penetrate the dextran layer. This will decrease sensitivity of the sensor chip, as not all immobilised ligand is available. The orientation of the bound erythrocyte is also unpredictable. Although the cell has a large diameter, it is only approximately 1.7 $\mu$m thick. Therefore one cell may cover a large area if lying down on the chip, or a small area if bound on its side.

In addition, Quinn *et al.* (1997) described the used of Concanavalin A (ConA) as a lectin that binds to all erythrocytes irrespective of blood group. ConA binds to surface glycoproteins through mannose and glucose residues and is isolated from the Jack Bean (Kornfeld and Ferris, 1975). ConA has a molecular weight of 25,500 per protomer (Becker *et al.*, 1975) although above pH 6.8 ConA exists as a tetramer with one binding site per monomer. Above pH 7.0 it forms larger aggregates. The molecule requires the presence of calcium and manganese ions for binding to glycoproteins (Becker *et al.*, 1975). ConA is used for the development of SPR procedures in this work and is also considered as a secondary detection molecule when fluorescently labelled.

### 1.3.2.6 Alternatives to Existing Solid-Phase Methods

Diagnostic testing systems and products are constantly evolving and improving. Any new system must offer benefits over the existing technology to be accepted. Smaller, faster and cheaper methods are most likely to succeed if they also offer high throughput, specificity, sensitivity and positive sample identification. New technology may offer the next generation of diagnostic testing systems. It is probable that superior technology will replace existing technology, even if initial costs are higher, as increased uptake tends to lower costs (Petrik, 2001).

In 1997, Quinn *et al.* demonstrated that the solid-phase method of SPR has the ability to determine blood groups. By the start of this work, data had also been published which stated that the microarray platform was suitable for protein applications. Therefore, there was potential that solid-phase assays such as microarrays could be

used for blood donation testing. Microarrays are already well established for DNA analysis. Microarray technology may potentially offer an alternative to the testing of one donation on many platforms where a donation could potentially be comprehensively characterised on one 'chip'. The expansion of these chips to include further tests as they become available or required is also considered relatively straightforward provided the appropriate reagents are available.

This attempt to develop this new technology is considered both timely and novel. Since the start of this work, the research in this field has advanced from limited published material to copious amounts available on many types of protein arrays. From literature searches performed thus far, this is the first and only work that looks into the possibility of comprehensive blood testing by protein microarray.

## 1.4 Microarray Technology

### 1.4.1 The Origins of Microarrays

Watson and Crick published the double-helical structure of DNA in 1953. This was the origin of the scientific field of molecular biology. Many methods used in molecular biology exploit the fact that nucleic acid strands bind by base-pair complementarity, where bases adenine-thymine (A-T) and guanine-cytosine (G-C) pair in DNA and adenine-uracil (A-U) and G-C pair in RNA. Traditional methods in molecular biology worked on a one gene – one experiment basis, and this meant that experimental throughput was limited. However, the great advantage of microarrays is the potential for massive parallel analysis; the technology of DNA microarrays allows information on thousands of genes to be studied simultaneously on one

microarray 'chip'. The term chip is used to describe microarrays, which are also referred to as slides or microchips, biochips, or more specifically as gene or protein chips.

A biological array is described as an orderly arrangement or collection of molecules (Petrik, 2001). A traditional array experiment may use common assay equipment such as microplates or blotting membranes, and may be prepared manually or make use of robotics to deposit samples. Array assays may be described as either macroarrays or microarrays, the difference being in the size and density of the spots. The Southern blot, mentioned below, may contain spot sizes of 300 $\mu$m or above. Microarrays may have a far greater density – one array can potentially contain thousands of spots.

It is generally accepted that the field of DNA microarray technology evolved from a discovery made in 1975 by Ed Southern (review, Lander, 1999). The Southern blot was one of the first kinds of arrays, where nucleic acid molecules are attached to a solid support and probed with labelled RNA/DNA molecules. In 1989, Ekins reported on the ambient analyte immunoassay and suggested the theory that a 'large range of individual immunoassays on a small immunoprobe' could be used to measure analytes in small samples. Although the Ekins theory is based on immunoassay rather than DNA assay, the basic assay approach of microarrays had been in existence for quite some time.

In 1995, a group from Stanford University published work (Schena *et al.*, 1995), which caused an explosion in the interest towards microarray technology (Lander, 1999). Rather than using porous membranes, they described the use of solid supports such as glass microscope slides on which thousands of nucleic acid molecules could be 'spotted' using pins. These spotted microarrays were then hybridised with a labelled probe, then scanned and analysed. Following this, high-density arrays soon became possible with advances in robotic printing technology.

In 1991, Fodor *et al.* described a method to produce arrays of 400,000 distinct oligonucleotides each within its own 20 $\mu$m area. This was achieved using photolithography-masking techniques that enabled the synthesis of the oligonucleotides at set positions. This innovation, along with that described by the Schena *et al.*, (1995) led to the explosion of interest of microarrays.

In the years between 1991 and 1995 other methods were being developed. Walt *et al.* (1993) were working with cDNA beads, which formed the basis of the Illumina system. Although beads offer advantages, the developments by Schena *et al.* made the most impact as it was more readily available technology.

In the literature there is a conflict of opinion within publications on the historical developments in the microarray field. Ekins and Chu (1999) criticise the publication by Schena *et al.* (1998) for its descriptions of origins of the technology, which they believe had been originated with the ambient analyte theory (discussed later).

In array technology, there are two frequently used terms; probe and target. This manuscript follows the nomenclature that a 'probe' is a molecule of known sequence or specificity (usually the immobilised molecule), and that a 'target' is a sample of which the identity, presence or abundance is unknown. **Figure 1.7** illustrates the simplest form of general microarray principles. A probe is first immobilised onto a solid surface. The surface is then blocked before incubation with a fluorescently labelled target. Options for each of these stages will be discussed later.

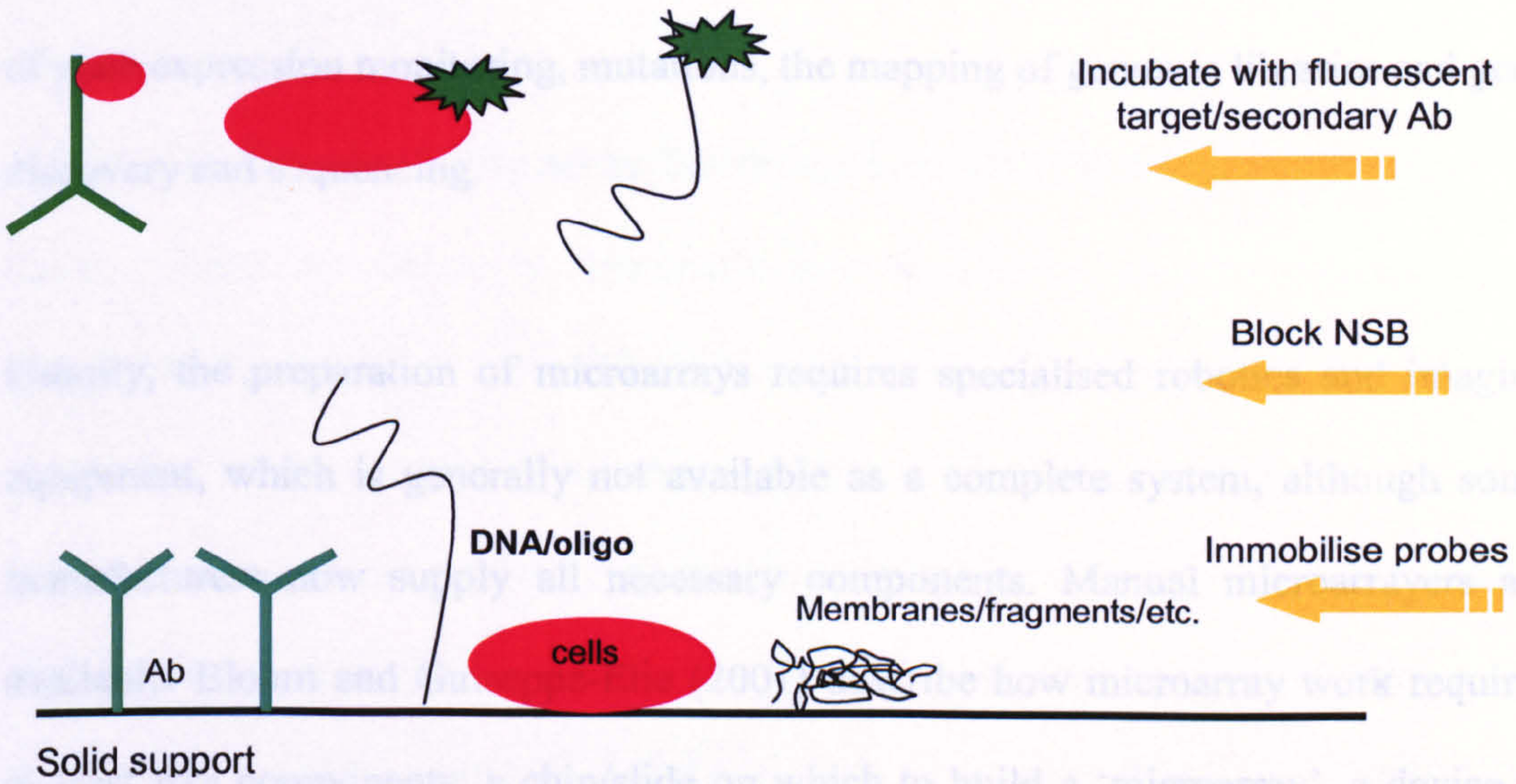



**Figure 1.7**. Schematic of simple microarray or solid-phase principles (not to scale).

Since the invention of microarray technology the applications have been widely explored. It is now possible to analyse all the genes of certain organisms at once (Blohm and Guiseppe-Elie, 2001). In 1998, Ramsay reviewed the state-of-the-art, which gave an overview of the microarray technology at that time. This paper also clarifies the two variations in the use of DNA microarrays: one where sample (target) DNA is immobilised then exposed to labelled probes before complementary

sequences are analysed. This is the method initially developed by the Stanford University group (Schena *et al.*, 1995). These microarrays have been used to monitor the expression levels of certain genes and for large-scale screening; secondly, oligonucleotide probes may be pre-synthesised and then immobilised or synthesised on the chip using light-directed synthesis (Fodor *et al.*, 1991). The array is then exposed to labelled target DNA and complementary sequences analysed. The on-chip synthesis developed by Fodor *et al.* is the method employed by Affymetrix (Santa Clara, CA) in their product, the GeneChip™. These chips have been used in the study of gene expression monitoring, mutations, the mapping of genomic libraries and gene discovery and sequencing.

Usually, the preparation of microarrays requires specialised robotics and imaging equipment, which is generally not available as a complete system, although some manufacturers now supply all necessary components. Manual microarrayers are available. Blohm and Guiseppe-Elie (2001) describe how microarray work requires at least five components: a chip/slide on which to build a 'microarray', a device to fabricate the microarrays, a system or method for hybridisation, a scanner to read the microarrays and software to process and analyse the results. This does not include equipment for the preparation of samples and probes. This paper also mentions the immense variability brought about by the various stages of the experimentation. Information on the building of a microarray system is available from the Stanford Microarray Database website and in Cheung *et al.* (1999).

### 1.4.2 Design Options for Microarray Experimentation

This section outlines experimental design considerations in the development and optimisation of a microarray experiment, based on the existing work in this field. There are several steps involved in the design and use of a microarray system, and many different options available within each step. **Figure 1.8** outlines the design considerations encountered in microarray development and can apply to both DNA based and protein based microarrays. These are discussed here and will be applied throughout this work.

#### 1.4.2.1 Options for Microarray Surfaces and Microfluidics

Glass microscope slides, if chemically treated, can be used to prepare arrays containing thousands of spots (Cheung *et al.,* 1999). Glass slides are ideal for the production of many types of arrays, especially DNA arrays, as they can withstand high temperatures, are relatively cheap and, as glass is non-porous, reaction volume may be kept to a minimum (Petrik, 2001). Glass slides are commonly coated with poly-L-lysine for the arraying of DNA as well as proteins (Haab *et al.,* 2001), but other coatings have been explored. There are alternatives to glass slides such as silicone, plastic and beads, which could be selected if appropriate to the assay. Alternative slide coatings are discussed below, but the options available are always increasing as research laboratories seek optimal probe immobilisation and novel surfaces.

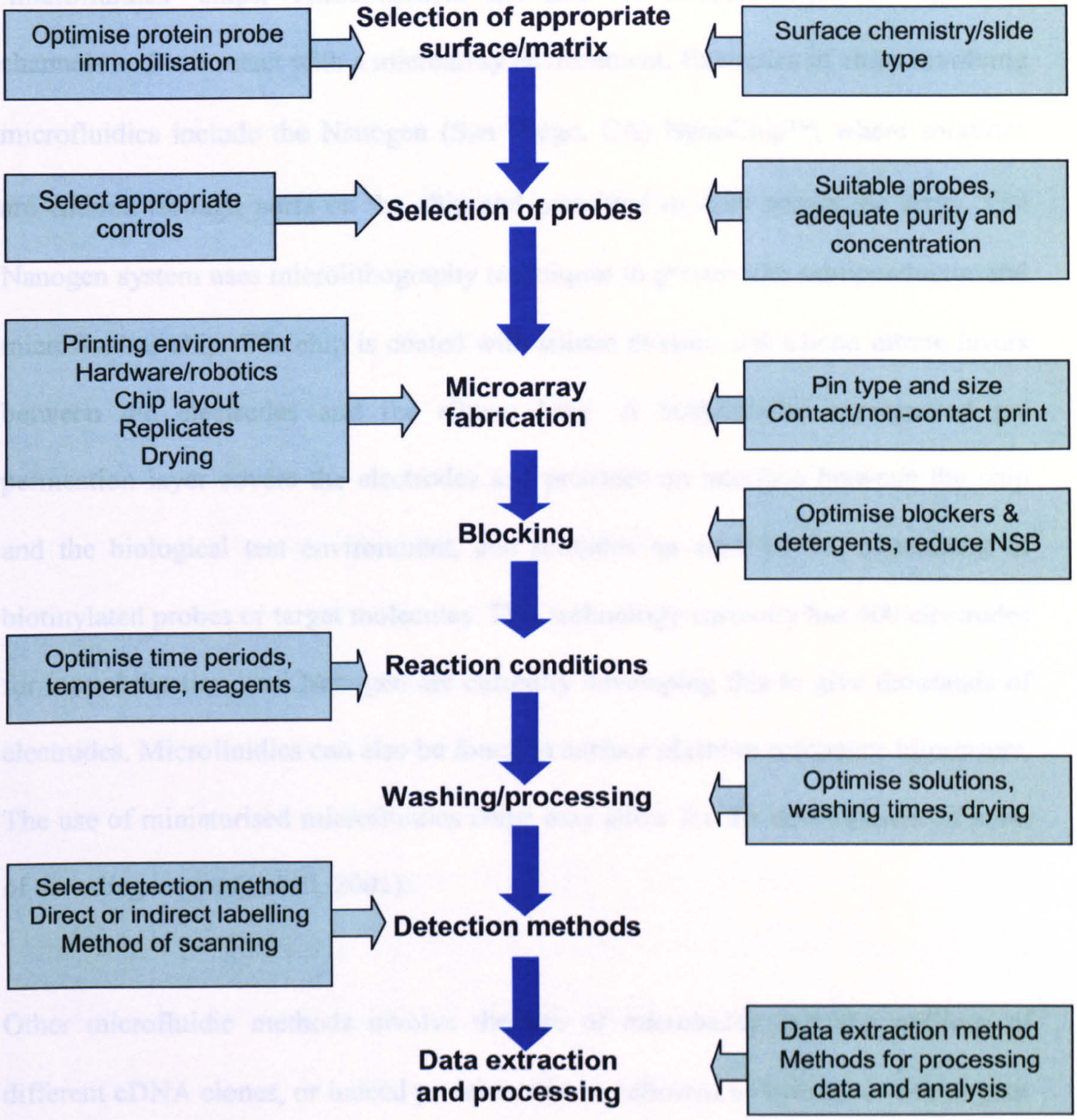



**Figure 1.8**. Design considerations in microarray development, experimentation and optimisation.

Recent advances in microarray technology have seen the development of 'microfluidics' chips. These involve the flow of samples/targets through fine channels and in contact with a microarray environment. Examples of chips involving microfluidics include the Nanogen (San Diego, CA) NanoChip™, where solutions are entered through ports on the chip and permitted to flow across the array. The Nanogen system uses microlithography techniques to prepare the semiconductor and microfluidics chip. The chip is coated with silicon dioxide and silicon nitrate layers between the electrodes and the silicon base. A streptavidin impregnated gel permeation layer covers the electrodes and provides an interface between the chip and the biological test environment, and provides an area for the attachment of biotinylated probes or target molecules. This technology currently has 400 electrodes for immobilisation, and Nanogen are currently developing this to give thousands of electrodes. Microfluidics can also be found in surface plasmon resonance biosensors. The use of miniaturised microfluidics chips may allow for the development of point of care diagnostics (Cahill, 2001).

Other microfluidic methods involve the use of microbeads carrying millions of different cDNA clones, or indeed proteins, that are allowed to hybridise with targets. An array is assembled in a flow cell and analysed by a fluorescent based signature sequencing method (Blohm and Guiseppe-Elie, 2001). This method can be used for measuring gene expression and more recently has been developed for use in protein studies. Companies including Illumina have developed systems such as these for protein studies. Microbeads have also been used to attach molecular beacons that produce a fluorescence signal after binding of unlabelled target molecules. A

chemical change allows the beacon to give off a particular detectable fluorescence signal.

Options for microarray surfaces, fabrication and immobilisation methods and detection methods are summarised in **Table 1.6** with some examples of each.

### 1.4.2.2 Selection of Probes for Spotting

Probes selected for experimentation must be of suitable purity, specificity and concentration. High purity is optimal due to small spot sizes and, therefore, reaction area. Proteins can bind to surfaces non-specifically, and the presence of contaminating proteins means there will be competitive binding which should be avoided especially on small surface area (Beck *et al.*, 1985). The reduction of unwanted substances may also reduce non-specific binding (NSB). The optimal concentration of the probes must be optimised, and it is important to use reasonably high concentrations with subsequent dilutions on the same chip until the optimal concentration is determined. At this point crucial control probes should also be considered. A microarray experiment without suitable positive and negative controls is statistically unsound. Negative controls are particularly important with regards to data analysis. In this work a data processing and analysis method was established which used the signal/noise ratio (S/N) to determine reactions. Negative controls are used to calculate 'noise'.

**Table 1.6.** Table outlining surfaces, fabrication/immobilisation and detection methods available, including non-exhaustive examples.

| Surfaces | Fabrication/Immobilisation Methods | Detection Methods |
|---|---|---|
| Glass/silicon/plastic slide substrate | Contact printing | Fluorescence (confocal scanning) |
| Polymer coating (poly-L-lysine, ZetaGrip) | - solid pins | Surface plasmon resonance (Biacore) |
| Metal coating (gold, silver) | - split pins/ pin and ring | Resonance light scattering (Genicon) |
| Gel coating (polyacrylamide) | Non-contact deposition (ink-jet) | Flow cytometry (Luminex) |
| Membrane layer (nitrocellulose) | In situ synthesis (Affymetrix) | Fibre optics (Illumina) |
| Derivitisation (aldehyde, epoxy, amino) | Affinity/covalent binding | Planar waveguide (Zeptosens) |
| Beads (polystyrene) | Microfluidics | Mass spectrometry MALDI (Sequenom) |
| Nanocrystals (Quantum Dot) | Electrode activation (Nanogen) | Mass spectrometry SELDI (Ciphergen) |

Although proteins in this work are immobilised only in PBS, the use of preservatives to increase protein stability should be considered when evaluating the potential of products with a practical shelf-life. Glycerol can be added to minimise dehydration and sugars can be added to improve antibody stability (Lee and Kim, 2002). MacBeath and Schreiber (2000) describe the use of 40 % glycerol, which would obviously affect protein concentration, and Lee and Kim (2002) describe how PEG 200 at concentrations of over 10 % can reduce evaporation and stabilise proteins. The different types of probes previously described or others with potential are discussed in section 1.4.3.

### 1.4.2.3 Microarray Fabrication

The density of a microarray depends on the spatial resolution of the robotics used (Sinclair, 1999). The value of the experiment depends critically on the quality of the printed array (Blohm and Guiseppe-Elie, 2001), which can be fabricated by a variety of methods. The most common method used is pin-transfer, although other methods include the in-situ synthesis method originally described by Fodor *et al.*, (1991) and ink-jet printing where there is no direct contact with the chip surface and has been used to reduce spot sizes to 25-30 $\mu$m (Okamoto *et al.*, 2000). Roda *et al.* (2000) describe the adaptation of a conventional ink-jet printer for use in printing proteins onto various sheet surfaces. Non-contact methods may give better quality of array printing. In bead-based microarray systems the probe is attached by chemical means.

Pin-transfer was developed by the Stanford group, and involves the use of a robotic arrayer to directly spot material onto the surface of a chip using metallic pins.

Various pin sizes may be used, with various spacing between the spots. The diameter and shape of the pins determines the volume spotted and the size of the spots. Solid pins, split pins or a 'pin and ring' method may be used. The pin method can sometimes result in irregular printing and slide-to-slide variation (Petrik, 2001) and if using solid pins the print head must return to the stock plate prior to each contact, taking longer to print an array. Care must be taken when using pins to ensure wearing is minimal as this can affect printing. 'Pin and ring' devices are available for sample spotting (Cortese, 2000a). The ring holds some of the arraying solution, the pin punches through this to deposit a small droplet onto the array surface. This system allows the deposition of many droplets without the print head having to return to the stock plate for array solution.

For pin-transfer, most instruments use pins attached to a print head on a simple XYZ axis robot and transfer the sample from a microtitre plate to the microarray surface. The quality of a microarray can be seriously affected by the reproducibility and shape of the pins. Spot quality may also be affected by the solution composition, the slide surface and the environmental conditions (Bacarese-Hamilton and Crisanti, 2002). Replicate spots must be considered, as this also is crucial in the soundness of statistical data.


**Figure 1.9.** The BioRobotics MicroGrid II Arrayer (biorobotics.com).

In **Figure 1.9** the BioRobotics MicroGrid II arrayer is pictured, which is capable of printing up to 120 K features per array. This arrayer uses typical XYZ robotics and was used extensively in the project presented in this thesis. **Figure 1.10** shows steel pins for use in deposition, and the print heads in which they are held and positioned. The microarray spotting system must also provide a facility for washing and drying pins between samples to avoid sample cross-contamination.

Washing of pins may be performed by shaking the pins in a cleaning solution (Lueking *et al.,* 1999), or in a sonicator or flowing water bath. Pin drying may be performed by the use of a vacuum (Cheung *et al.,* 1999).

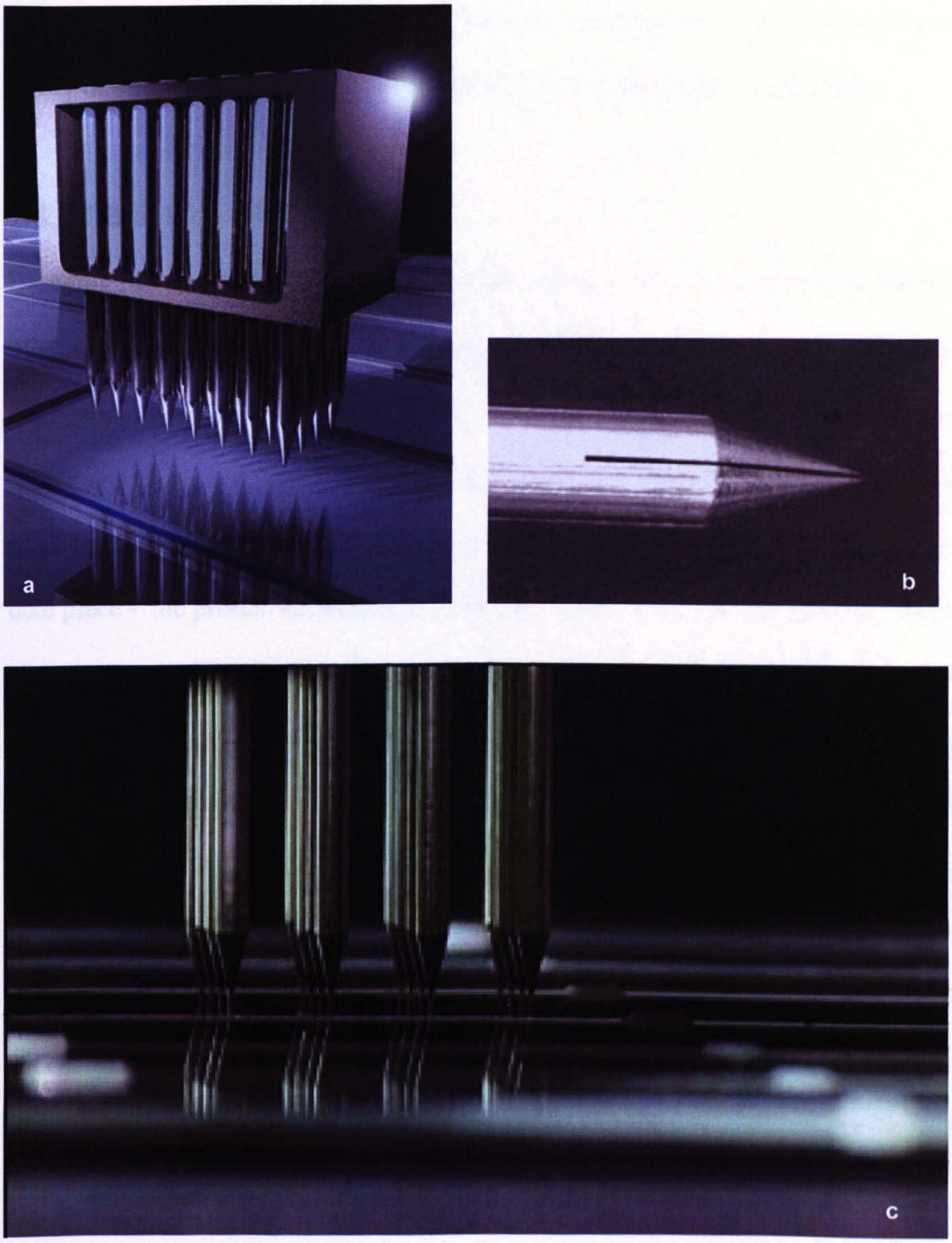



**Figure 1.10.** Photographs showing steel pins for fabrication of microarrays. Figure 1.10a shows 32 pins spring-loaded in a print head for robotic printing. Figure 1.10b shows a split pin. In Figure 1.10c a 16-pin formation is seen printing onto microarray slides (photographs from combichemlab.com and microarray.uc.edu).

Spotting should be performed in a clean but humid environment to prevent clogging of pins with dust and the evaporation of samples. After printing, the slides should be

left for sufficient time as to allow the spots to air dry. Cortese (2000a) reviews the 'array of options' available within this field with regards to instrumentation.

### 1.4.2.4 Blocking of Microarrays

Blocking of microarrays must be performed to block sites where non-specific binding (NSB) may take place. In protein microarrays, blocking is usually performed using appropriate levels of milk protein or bovine serum albumin, in the presence of buffer and detergent. The introduction of non-target protein onto the assay surface prior to the addition of specific target molecules allows NSB of protein-protein to take place – the protein molecules effectively bind to and block any potential binding sites that may allow subsequent non-specific interactions. A detergent will compete with protein for hydrophobic sites on the surface, therefore reducing NSB (Matson, 2000). Detergent should be at a low concentration as it may displace the desired protein probes from the surface (Matson, 2000). Therefore, an optimal blocking solution should minimise NSB, without reducing specific binding. To increase specificity and reduce NSB, it is of extreme importance to optimise the blocking of any assay. To reduce NSB further the blocking agent can also be included in the target solution. The use of different blockers and detergents will be explored in this thesis (see Chapter 3).

### 1.4.2.5 Microarray Reaction Conditions

The reaction conditions of the microarray have to be optimised throughout the development process. Considerations are:

- **Target solution.** The target must be of suitable concentration to be detected. It may or may not be diluted into blocking buffer. The volume of the target solution must be optimised considering concentration and likelihood of probe-ligand interaction. Particular thought is required when dealing with blood samples due the presence of plasma proteins.

- **Time of incubation.** As with all biological assays, the time allowed for interaction can greatly influence the outcome of the assay. Here the optimal time period would be as short as possible while still allowing suitable sensitivity.

- **Temperature.** Optimal temperature can affect results and the interaction of probe and target. Temperature is critical in the assay of clinically significant blood tests.

Specific microarrays will require further optimisation e.g. blood typing microarrays could be affected by probe concentrations, ionic strength of solutions and pH.

### 1.4.2.6 Washing and Processing of Microarrays

Following incubation with target solution microarrays, like many other types of assay, require a washing step to remove unbound materials. An optimal processing method will remove unbound material whilst limiting removal of specifically bound targets. The procedure may also help in the reduction of NSB and, therefore, reduce background noise. Phosphate buffered saline is used commonly in protein assays and

in early examples of protein microarray publications. Detergents may also be included to optimise conditions.

### 1.4.2.7 Microarray Interaction Detection Methods

Existing microarrays commonly use 'fluorophores' for detection of binding. The actual fluorescent label is not coloured, but when excited by a specific frequency of light by a laser, a detector measures the light emitted from the label. Only light of this specific wavelength will cause the fluorophore to emit a characteristic emission wavelength that is different from the excitation wavelength. The detector must filter out the excitation wavelength and measure only the emission. If using two fluorophores in an experiment, they must have distinct emission or excitation wavelengths. Two or more 'fluorophores' may be used for simultaneous analysis of multiple test results or 'multiplexing'. The detector resolution used must allow reading of fluorescence of the spot size used in the assay.

Microarrays require fluorescence scanning to extract the results. Confocal laser scanning is used to provide high image and data quality. Confocal means that there are two places where light is in focus. Excitation light is focused at the microarray surface and emission light is focused at a pinhole. The use of the pinhole restricts the depth of focus therefore detecting fluorescence from a limited depth at the surface. This means that out of focus light from unwanted artefacts, such as dust on the microarray surface, is reduced. The microarray is 'scanned' using mirrors that reflect the laser light across the slide. Light that gets through the pinhole is detected by a

photomultiplier tube (PMT), and the image is compiled from the scan. The voltage to the PMT can be altered to increase or decrease the image intensity.

Cy3™ and Cy5™ are commonly used as the fluorophores in microarray experiments as they are intensely fluorescent. These are cyanine NHS-esters that bind to free amino groups. The emission wavelength of Cy3™ is 570 nm and Cy5™ is 670 nm. As an alternative it is possible to use Fluorescein Isothiocyanate (FITC) as a fluorophore, and generally this may be detected using the same scanning settings used for Cy3™.

In a protein microarray, ideally the target solution would be labelled. However, this may present problems in blood typing arrays where erythrocyte or antibody is the target. A sandwich type assay is one option where the fluorescent label is attached to another protein or antibody that will bind to all erythrocytes or human antibodies attached to the array. This could be a one or a two-step assay where the red blood cell is pre-labelled with the fluorescent protein. The compatibility of probe and detection methods must also be considered. For example, a human/mouse monoclonal antibody probe would be likely to bind an anti-human Ig antibody if used for detection and potentially give false positive results.

Interactions on protein microarrays have been demonstrated by a variety of techniques already mentioned. In some assays it may be necessary to amplify the detection signal to allow more sensitivity in the detection of weak targets. Tyramide deposition and fluorophores such as cyanine dyes have been described to increase

sensitivity of detection in microarrays. Rolling circle DNA amplification (RCA) has been applied to improve sensitivity in immunoassays, including microarrays. In 'immunoRCA' (Schweitzer *et al.*, 2000) a primer is attached to the secondary antibody molecule by biotin-streptavidin interaction or covalent linkage. In the presence of DNA polymerase and nucleotides, and at isothermal temperature, circular DNA is allowed to hybridise to the primer and amplifies it by RCA. A label is added which hybridises with the tag resulting in a long DNA molecule with the detection label built in. This method is reported to increase signal greatly over non-amplified signals, and may be ideal for an open-plan microarray environment.

### 1.4.2.8 Data Analysis

Data from microarrays is quantified by measuring the fluorescence intensities of individual spots. Usually, computerised image analysis is performed by placing a grid over the array area and positioning imaging optics to the centre of the spot. A background signal for each spot is measured from around each spot on the chip surface. Several factors can give a poor signal to background noise ratio; fluctuations in light source, poor blocking and therefore NSB, scattering of fluorescence from other spots and other markings on the chips such as dirt or dust which must be avoided. The processing method is critical in the elimination of these factors.

One of the greatest challenges encountered in microarrays is analysis of results. The sheer volume of data generated by a high-density microarray can be intimidating and requires much manipulation (Lander 1999), and methods of analysis have to be well developed to acquire meaningful data (Forster *et al.*, 2003). Siedow (2001) reported

from a meeting devoted entirely to the evaluation of methods for analysing the large quantities of data generated from gene expression microarrays. Replication is valuable due to chip to chip variability and variability in sample preparation and gives a far better sense of data variability.

Blohm and Guiseppe-Elie (2001) gives an overview, at that time, of the many uses of DNA microarrays. While the original microarrays were for DNA based analysis, the evolution of the technology to work with proteins and other biological molecules had been apparent.

### 1.4.3 Protein Microarray Development

#### 1.4.3.1 The Requirement for Protein Microarrays

While protein microarrays are built on the same principles as DNA microarrays, their use brings about new challenges in all aspects of the technology due to their structural and functional requirements. The number of publications on DNA microarrays far outweighs that of protein microarrays, although these have steadily increased in the course of this work. Siedow (2001) commented that the literature in DNA microarray field is so far ahead of the reality in 2001, yet this could not be said of protein microarrays when this work was initiated. The delay in protein microarrays compared to the advent of DNA microarrays may be partly due to problems with protein stability, immobilisation chemistries and weaker detection signals (due to lack of amplification) (Bacarese-Hamilton *et al.*, 2002), and the range and complexity of protein targets.

The main driver for protein microarrays was to bridge the gap between genomics and proteomics and the study of protein function - not immediately for diagnostics, although this potential was realised once the technology began to evolve. Diagnostics could be the main area where protein microarrays are introduced in the near future (Cahill, 2001). Proteomics is the study of identification and characterisation of proteins in biological systems to assign function (McWhirter, 2001), the term proteome referring to all proteins expressed by a genome. Aspects such as expression, post-translational modifications and interactions with other substances can be studied. Nucleic acid based technologies such as PCR and microarrays provide important genomic results, yet there is a lack of correlation of gene expression and mRNA levels, with the amount of protein expressed in the cell (Dalmasso, 1999; Borrebaeck *et al.,* 2001). This may be caused by different rates of translation from mRNA, post-translational changes that cannot be identified by DNA arrays, as well as protein degradation by proteolysis (de Wildt *et al.,* 2000; Kusnezow and Hoheisel, 2002). Where DNA can be immobilised and denatured (Schena *et al.*, 1995) proteins have secondary and tertiary structures that have to be maintained for functional analysis, and can be prone to denaturation if exposed to an unsuitable environment.

Traditional techniques for the study of proteins include one and two-dimensional gel electrophoresis, sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE), liquid chromatography, mass spectrometry, ELISAs and blotting. Where traditional methods meant limited amounts of assays could be run at any time, the microarray era has the potential to change this for protein arrays as it has for DNA

arrays (Borrebaeck, 2000). The use of DNA microarrays has demonstrated the ability to run thousands of assays in parallel.

The development of protein microarrays has immense implications to the field of immunoassays, given that antibody-antigen interactions can be analysed in a similar format to microplate ELISAs but with far greater throughput, convenience and reduced costs (Bacarese-Hamilton and Crisanti, 2002). The technology was being explored as a potential replacement to ELISAs, such as the work performed by Bacarese-Hamilton *et al.* (2002). The first indication that miniaturised amounts of immobilised antibody could be used in a ligand binding assays may have come from Ekins (1989) who described the ambient analyte assay theory. Here, capture antibodies were immobilised as a microspot on a solid support and used to measure analyte concentration. Bound analyte could be measured using labelled molecules. However, it is probable that although the theory had been demonstrated, the potential for large-scale protein microarrays arose only as a result of genomic microarrays. Kusnezow and Hoheisel (2002) give a thorough overview of protein microarrays and discussed some of the promises and problems associated with the development of such arrays.

### 1.4.3.2 The Development of Protein Microarray Technology

Once the theory of microarrays was met by the potential to fabricate and process microarrays, publications on the use of protein microarrays began to emerge. Assay miniaturisations on microarray formats were being published by the late 1990s. Mendoza *et al.* (1999) attempted antibody arrays to replicate standard ELISAs. Filter

membranes were also being used as they were the standard for protein studies (Kusnezow and Hoheisel, 2002). Ge (2000) published work using nitrocellulose membranes and manual dot-blot apparatus to produce low-density protein arrays for quantitative detection of protein interactions with various ligands, using autoradiography for detection. Huang (2001) and Huang *et al.* (2001) described antibody based protein microarrays for detection of proteins, and specifically cytokines, in solutions and patient serum. de Wildt *et al.* (2000) used filter-based ELISAs to screen phage-antibody libraries, where clones were robotically picked and gridded onto an antigen coated filter in a mass array; protein L-horseradish peroxidase was used for detection of bound antibody fragments. Filter based arrays tend to be low resolution and have high background values (Kusnezow and Hoheisel, 2002). The method described by de Wildt *et al.* can potentially be used for monitoring protein expression, using phage-displayed antibodies to proteins under study. Unlike conventional 2-D gel electrophoresis, the filter array method can be used to analyse the expression of more than one protein in a source material at one time.

The move to glass slides for preparation of protein microarrays was demonstrated by Guschin *et al.* (1997) and Arenkov *et al.* (2000) who described methods for producing protein arrays using gel coated glass slides. Arenkov *et al.* (2000) accelerated their immunoassay using microelectrophoresis, during which the fluorescently labelled solution was added. The benefit of using the gel was that it gave low background, low non-specific binding and good support for immobilisation of proteins while retaining biological activity. These techniques have been

successfully employed in preparation of both antibody and antigen arrays. They immobilised recombinant hepatitis B virus antigen and showed detection of FITC labelled antibodies purified from hepatitis B positive human serum. The system they developed could accommodate proteins up to 400 kDa. This would be limiting in the field of blood typing diagnostics, as the standardised antibodies are IgM (970 kDa approximately).

Much of the prior-art in protein microarrays uses solid pin printing as opposed to pin and ring or inkjet deposition. Solid pins may be preferable due to the viscosity of protein solutions, which may clog alternative systems (Bacarese-Hamilton and Crisanti, 2002).

Lueking *et al.* (1999) described the use of polyvinylidene difluoride strips to immobilise proteins for the purpose of screening monoclonal antibodies against whole libraries of proteins. This technology could be adapted to be useful in the future development of monoclonal antibodies, to aid the isolation of antibodies with specificity of interest. Secondary antibodies could also be used to screen for antibodies of certain class or subclass depending on the requirement.

### 1.4.3.3 Antibody Microarrays

Antibodies are ideal for use in protein microarrays due to their specificity and structure, but they must be immobilised without affecting function. Globular proteins tend to unfold and lose activity when attached to a solid support (Borrebaeck *et al.*, 2001). The orientation of antibody molecules onto a solid surface has implications

with regards to the accessibility of the antigen binding sites. Malmsten (1995) reports that the Fc portion of an IgG molecule is more readily adsorbed onto a solid surface than the Fab portion. This may be because Fab is more structurally stable than the Fc portion (Hlady and Buijs, 1996) making the Fc more susceptible to adsorption to a surface. If the molecule binds via the Fc portion, it follows that the Fab region is more likely to be orientated in a favourable way to enable interaction with antigen.

Using robotic instrumentation adapted from DNA microarrays, MacBeath and Schreiber (2000) developed high-density protein microarrays covalently attached to glass slides coated with aldehyde reagents. In parallel to the field of DNA microarrays, Haab *et al.* (2001) printed high-density protein microarrays onto poly-L-lysine slides (electrostatic adsorption) and used fluorescently labelled targets (antibodies or antigen) to detect antibody-antigen interactions. This meant that the equipment, software and experimental approaches were broadly similar to those used in DNA microarrays and opened the microarray field for high throughput protein microarrays.

Belov *et al.* (2001) used an antibody microarray to investigate and characterise antigens on leucocytes and leukaemia cells and to determine antigenic expression, or immunophenotype of different malignancies. FITC conjugated secondary antibody was used for detection using a confocal microscope. This was the first publication in which antibody microarrays were shown to bind whole cells.

Li *et al.* (2003) used anti-cytokine arrays for the detection of cytokines, and performed a comparison of direct and indirect cytokine labelling. Their purpose is to develop anti-cytokine arrays for profiling cytokines in subcutaneous wound healing tissue.

Where antibodies are not available or antigen not isolated, other probes can be used. Phage-antibody libraries can provide specificity to almost any antigen (de Wildt *et al.*, 2000), where the phage expresses antibody Fab or single chain variable fragments (scFv) on its surface. Phage libraries use a genetic framework and attach various germ-line genes representing heavy and light chains. Some phage libraries can represent about $10^{11}$ to $10^{12}$ different antibodies. Zyomyx (Hayward, CA, USA) manufacture high-density microarrays of phage-display libraries.

### 1.4.3.4 Antigen Microarrays

Where antibody microarrays can be used to test for the presence of antigen, antigen arrays do the reverse. These can be used to check for presence of antibodies. Antigen microarrays may be used many purposes such as the determination of autoimmune disorders (Robinson *et al.*, 2002) or for determination of serum antibody titres to pathogens (Ewart *et al.*, 2001). Joos *et al.*, (2000) demonstrated that high titres of antinuclear antibodies were detectable against nuclear proteins and nucleoprotein complexes in patients suffering from forms of rheumatic disease when analysed on an antigen microarray. The ability to diagnose disease has wide implications in immunodiagnostics, biotechnology, drug discovery and vaccine preparation (Cahill, 2001).

Seong (2002) discusses the immobilisation of antigens for use in immunodiagnosis of infectious diseases and the paper reviews methods for optimisation of proteins for this purpose. The use of silylated, silanated and epoxy coated slides was explored to immobilise human IgM, human IgG and biotinylated Protein-L probes at different pH. A biotin-streptavidin detection method was employed. Seong (2002) reports that the use of a spotting buffer at a pH that induces an overall negative charge may help increase immobilisation of proteins to a positively charged surface.

Any antigen which can be immobilised to the solid surface and retain its functional state can theoretically be used in an antigen microarray. Carbohydrate antigens can be immobilised, and as these are present on the surface of most cells they can be the target of molecular markers such as antibodies. This is true of the ABO blood group system antigens. Wang *et al.* (2002) demonstrated microarrays of carbohydrates, and their use in binding antibodies from serum. They immobilised several polysaccharides onto nitrocellulose coated slides and these were detectable by target IgG and IgM antibodies. As carbohydrate expression can also vary in disease states such as cancer, retroviral infection and in various stages of cell development, these could be useful microarray probes. Fukui *et al.* (2002) also demonstrated carbohydrate interaction studies on microarrays. This is of importance to proteomics due to protein glycosylation following translation.

Angenendt *et al.* (2003) describe a multiple spotting technique (MIST) in which target samples are spotted directly onto probes already on the microarray. This relies on the same robotics used for spotting the original probes being precise, to spot again

onto the same co-ordinates. The use of this system allows much reduced sample consumption and allows multiple samples to be evaluated on one microarray surface. The same group (Angenendt *et al.*, 2004) later used this format to evaluate and find specific recombinant antibody fragments.

Aptamers are short sequences of DNA or RNA with protein binding properties and subsequently may be used to bind their targets in relevant assays. Their binding of proteins relies on the formation of unique arrangements of hydrogen bonding (Reddy and Perrotta, 2004) and they bind similarly to antigen-antibody interactions (Lee and Walt, 2000). Aptamers can be produced by a process called systemic evolution of ligands by exponential enrichment (SELEX) (Tuerk and Gold, 1990). The SELEX process involves alternate cycles of ligand selection and amplification, which results in the enrichment of the desired sequence. Stadtherr *et al.* (2005) described an aptamer-based protein biochip, capable of capturing analytes in solution at the same sensitivity as antibodies to the same specificity. The engineering of microbial proteins to obtain antibody-like properties has also been developed as another way of obtaining antibodies (Kusnezow and Hoheisel, 2002). Peptides representing epitopes of antigens can be used and have been shown to detect protein kinase activity by Houseman *et al.* (2002).

Million *et al.* (1998) and Sinor *et al.* (1992) have demonstrated the immobilisation of erythrocyte membranes on microplates for antibody screening. Million *et al.* prepared membranes and then immobilised using paraformaldehyde. They used a

detection system of coloured antibody coated latex particles. This method could potentially be applied to microarray.

Another approach closely related to this thesis is that membrane proteins of cells can be isolated and spotted to prepare membrane protein microarrays. Fang *et al.* (2002a) published the first work on fabricated membrane protein microarrays consisting of membrane bound G protein coupled receptors on aminopropylsilane coated slides. They found that the use of aminopropylsilane slides gave good retention of the probes. The ability to print cell membrane proteins means that drug screening can be performed, as many drug targets are membrane-bound. Cheng *et al.* (2000) report that membrane proteins can be studied by incorporation into artificial lipid bilayers attached to a solid support. Letarte *et al.* (2005) used leucocyte membrane proteins as probes on a microarray and used them to detect antibodies and ligands to the proteins. It is thought that erythrocyte membrane proteins or membrane fragment arrays will be more stable in a microarray format than whole cells, due to their limited lifespan.

Very recently, Chan *et al.* (2004) demonstrated the preparation of whole cell lysate arrays. These were detected using specific primary antibodies followed by secondary antibodies conjugated to horseradish peroxidase. The signals were then amplified using tyramide-biotin conjugate, which is then reacted with fluorescently labelled streptavidin.

Tissue arrays have been prepared for molecular profiling of tumour specimens – samples from paraffin biopsies have been arrayed onto paraffin blocks and then analysed with appropriate reagents. This can be used to analyse tumours from different patients at different disease stages and for possible therapeutic use (Cahill, 2001).

### 1.4.3.5 Diagnostic and Commercial Protein Microarrays

There is so much diversity and competition in the field of diagnostics that there are currently companies world-wide involved in research, development and implementation of microarray systems for very diverse applications (Blohm and Guiseppe-Elie, 2001). In order for the implementation and uptake of microarray technology outwith research laboratories, systems will have to prove themselves robust, specific and sensitive, competent, reproducible and cost effective in comparison to existing methods. The commercialisation of a blood typing microarray will form part of the discussion of this thesis. An overview of companies active in the protein microarray field is presented in **Table 1.7.**

Ciphergen Biosystems Inc. (Palo Alto, CA) was one of the first companies to commercially enter into the protein array field (Cortese, 2000b) with its ProteinChip™ system. This system offers a range of surface chemistries for immobilisation and subsequent analysis. Interactions on the chip surface are read by surface enhanced laser desorption/ionisation time-of-flight mass spectrometry (SELDI-TOF-MS), so there is therefore no requirement to fluorescently label proteins. This approach determines the molecular weight or mass of the bound

material. This process can differentiate between expression of proteins in diseased and normal states (Dalmasso, 1999). The chips have been used in protein composition studies, target and marker protein discovery and identification, protein-

**Table 1.7**. Companies actively involved in the protein microarray field.

| Company | Website Information | Product Description |
|---|---|---|
| Affitech | affitech.com | Novel antibody production |
| Affibody | affibody.com | Antibody-like ligands |
| BD Biosciences (Clontech) | bdbiosciences.com | BD Atlas products |
| Biacore | biacore.com | SPR |
| Biosite | biosite.com | Triage protein chip |
| Caliper Life Sciences | caliperls.com | LabChip |
| Ciphergen | ciphergen.com | ProteinChip |
| Cambridge Antibody Technology | cambridgeantibody.com | Phage-display libraries |
| Domantis | domantis.com | Antibody arrays |
| Dyax | dyax.com | Phage-display libraries |
| Greiner Bio-One | greinerbioone.com | HTA platform |
| HTS Biosystems | Htsbiosystems.com | SPR arrays/Chemiflex™ platform |
| Illumina | illumina.com | BeadArray |
| Luminex | luminexcorp.com | xMAP technology |
| PerkinElmer (Nextgensciences) | lifesciences.perkinelmer.com | Protein array workstation |
| Phylos | phylos.com | PROfile™ chip |
| ProLinx | prolinx.com | Versalinx protein microarray |
| Schleicher & Schuell BioScience | sands.com | Cytokine arrays |
| Sense Proteomic | senseproteomic.com | COVET functional protein arrays |
| Somalogic | somalogic.com | Aptamer arrays |
| Zeptosens | zeptosens.com | Planer wave guide technology |
| Zyomyx | zyomyx.com | Silicon based antibody arrays |

DNA interactions, antibody capture, peptide mapping and sequencing. While it is similar to other solid-phase methods to study protein-protein interactions it cannot assess the interactions in real-time, in contrast to the technology of surface plasmon resonance.

Illumina (San Diego, CA, USA) have developed BeadArray™ technology for use in their Sentrix® system. They now sell the Whole Genome Genotyping BeadChip to analyse over 100K SNPs on one single microarray. The technology allows one million SNP assays per day (Oliphant *et al.*, 2002). The system uses a fibre-optic bundle as a substrate for microarrays. The end of each fibre is etched to create a well that can hold a 3 $\mu$m bead, each bead coated in many copies of the probe of interest – a variety of surface chemistries are available for the attachment of DNA, RNA and proteins. 50,000 fibres are fused together to create an array 1.4 mm in diameter. Bead libraries can be prepared and then exposed to the fibre bundle onto which they are randomly ordered. The location of each bead is identified after assembly. The system detects the presence of fluorescent targets by the use of laser excitation beams being passed through the fibres and the detection of emitted fluorescence. This technology has been used to perform blood group genotyping (Hashmi *et al.*, 2005) and has potential for a range of protein applications.

Luminex market the Luminex 100™ System, which is a multi-analyte profiling assay known as xMAP® technology. This uses internally dyed polystyrene microspheres with two different fluorochromes mixed in ratios to give distinct spectral addresses for each set. Probes are attached to the outside of the microspheres and then

incubated with target solution. A third fluorochrome is coupled to a reporter molecule and is used to quantify the interaction at the microsphere surface. The microspheres then pass through in a rapid stream and are interrogated by two lasers (similar to flow cytometry), which classifies each microsphere and also quantitates the reaction. Many different probes can be attached to the microspheres using a variety of surface chemistries and the technology has been used to perform immunoassays. 100 different microspheres can be screened simultaneously against one sample. This system requires the addition of secondary biotinylated detection molecule (e.g. an antibody) and then streptavidin conjugated indicator molecule, therefore extra steps are involved and reagent quality is of high importance, although the technique does seem rapid (20 minute estimated, Luminex.com).

Planar waveguide technology for microarray fluorescence imaging has been developed by Zeptosens. This utilises a special coating of $Ta_2O_5$ to induce an evanescence field for efficient fluorescence detection. This allows surface confined detection and dramatically cuts background noise interference, as only bound fluorescent molecules are measured (similar to SPR) and means that washing steps are not required (Pawlak *et al.*, 2002). This technology is part of a fully automated high-throughput protein microarray system. Pawlak *et al.* (2002) used this system with a hydrophobic surface to immobilise antibodies to human cytokines using ink-jet technology. Target samples were preincubated with Cy5™-streptavidin before incubation with the array, but no wash steps were required. This system offers advantages as there are fewer wash steps and the detection method is reported to be more sensitive than standard confocal scanning. Vörös *et al.* (2003) used the

Zeptosens detection system with PEG-Poly-L-lysine brushes to immobilise DNA, or if conjugated to biotin to attach streptavidin conjugated antibodies.

Based on knowledge gained and reading undertaken, at the outset of this work there was no work published on microarrays for comprehensive blood testing by the methods reported herein.

## 1.5 Project Aims

- To investigate a variety of methods for the preparation of antibody and antigen microarrays and the parameters important to study protein-ligand interactions in a microarray format.

- To establish optimal conditions for non-agglutination blood group determination on a microarray.

- To evaluate suitable methods of data acquisition, processing and analysis.

- To attempt to develop a reverse format assay for erythrocyte alloantibody screening.

- To verify and compare obtained data with the established analytical solid phase Biacore system.

# CHAPTER 2

# MATERIALS AND METHODS

## 2. MATERIALS AND METHODS

### 2.1 Sources

The composition of all buffers and chemicals used are given in **Table 2.1.** All chemicals were obtained from Sigma-Aldrich (Poole, U.K.) unless otherwise stated, and all were of reagent grade or higher. All water used was prepared by reverse osmosis unless stated otherwise. All probes and targets used are detailed in **Table 2.2.** Contact information for all suppliers used is listed in Appendix 1.

**Table 2.1** Buffer and chemical preparations used, with details of final concentrations and pH (where appropriate).

| Buffer/chemical name | Preparation |
|---|---|
| Acetic acid 0.3 % | Concentrated glacial acetic acid diluted to 0.3 % (52.2 mM) in purified water |
| Agarose 1 % | 1 % agarose diluted in 0.125 M Tris (hydroxymethyl) methylamine (Tris)(BDH), 0.385 M NaCl, 2.2 mM polyethylene glycol, 38 mM sodium azide ($NaN_3$). |
| Array Buffer 1X | Array Buffer 2X (Schleicher & Schuell) diluted 1 in 2 in PBS (pH 7.0) |
| Biacore running buffer | 10 mM HEPES, 0.15 M NaCl, 0.005 % P20 (Biacore), pH adjusted to 7.4 using 1 M NaOH |
| Biacore running buffer-Mn/Ca | 1 mM MnCl and 1 mM CaCl in Biacore running buffer. |
| Bicarbonate buffer pH 9.0 | 2.2 mM sodium bicarbonate (BDH), 2.2 mM sodium carbonate (BDH) |
| Bicarbonate buffer pH 9.3 | 0.206 M sodium bicarbonate, 0.081 M sodium carbonate. |
| Bicarbonate buffer pH 9.6 | One carbonate-bicarbonate buffer capsule diluted in water, to give 0.05 M buffer. |
| Crowle's double stain | 0.87 M glacial acetic acid, 0.184 M trichloroacetic acid, 4.3 mM crocein scarlet, 0.18 mM brilliant blue R, filtered before use. |
| ELISA blocking buffer | 10 % Marvel (Premier Brands Ltd, U.K.) in ELISA wash buffer. |
| ELISA coating buffer | 0.02 M Tris pH adjusted to 9.0 using 1 M HCl. |
| ELISA wash buffer | ELISA coating buffer plus 0.05 % Tween 20. |
| Ethanolamine | 1 M ethanolamine-HCl pH 8.5 (Biacore). |

| | |
|---|---|
| Ethanolamine pH 10.4 | 0.02 M ethanolamine, pH adjusted to 10.4 using 1 M NaOH. |
| FITC ConA 0.1 mg/ml | 0.1 mg/ml FITC Concanavalin A diluted in PBS. |
| Fixing solution | 50 % methanol, 7 % concentrated acetic acid, 43 % water. |
| Glycine-NaOH pH 9.8 | 25 mM glycine, 14 mM NaOH. |
| HCl 1 M | 1 M HCl. |
| HCl pH 1.5 | pH of purified water adjusted to 1.5 using 1 M HCl. |
| Methylated spirits | 70 % (final concentration) methylated spirits in purified water. |
| NaOH | 5 M stock solution prepared in water, from which dilutions were made. |
| NHS/EDC | 0.05 M N-hydroxysuccinimide, 0.2 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. |
| PBS | 0.15 M sodium chloride, 2.632 M Phosphate Buffer Stock Solution (Alba Bioscience), pH 7.0. |
| PBS pH 7.4 | 0.137 M NaCl, 2.7 mM potassium chloride, 1.5 mM potassium dihydrogen orthophosphate, 6.5 mM di-sodium hydrogen orthophosphate. |
| PBS-azide | 150 mM sodium azide in PBS pH 7.4. |
| PBS-BSA | 1 % bovine serum albumin (BSA) (ID Bio, France) in PBS. |
| PBS-BSA 2 % | 2 % BSA in PBS. |
| PBS-Ca/Mn | 1 mM MnCl and 1 mM CaCl in PBS. |
| PBS-milk | 3 % Marvel (Premier Brands Ltd, U.K.) in PBS. |
| PBST | PBS, 0.1 % Tween 20. |
| PBST-BSA | PBS-BSA, 0.1 % Tween 20 |
| PBST-milk | PBS-milk, 0.1 % Tween 20. |
| ProSep elution buffer | 0.1 M citric acid, pH adjusted to 3.0 using 5 M NaOH. |
| ProSep wash buffer | 0.15 M sodium chloride, 1 M glycine, pH adjusted to 8.6 using 1 M NaOH. |
| RBC-ID Diluent 2 (0.8 %) | Erythrocytes suspended to haematocrit of 0.8 % in ID-Diluent 2 (DiaMed). |
| RBC-ID Diluent 2 (5 %) | Erythrocytes suspended to haematocrit of 5 % in ID-Diluent 2 (DiaMed). |
| RBC-PBS | Erythrocytes suspended to haematocrit of 2 % in PBS. |
| SDS-PAGE running buffer | NuPage MES SDS Running Buffer 20X (Invitrogen) diluted 1 in 20 in water. |
| Sodium acetate | 1 M sodium acetate stock solution, pH adjusted using acetic acid, dilutions made in purified water and degassed. |

| | |
|---|---|
| Wash/Block buffer 1X | FAST PAK Protein Array Buffer 10X (Schleicher & Schuell) diluted 1 in 10 using purified water. |

**Table 2.2** Details of all probes and targets used, including cell line if monoclonal.

| Probe/Target Identification (cell line) | Protein Description | Source |
|---|---|---|
| Anti-A(B) (ES15) | Mouse monoclonal IgM antibody against blood group antigens A and B (reacts weakly with B) | Alba Bioscience |
| Anti-A (ES9) | Mouse monoclonal IgM antibody against blood group antigen A | Alba Bioscience |
| Anti-A (LA2) | Mouse monoclonal IgM antibody against blood group antigen A | Alba Bioscience |
| Anti-B (LB2) | Mouse monoclonal IgM antibody against blood group antigen B | Alba Bioscience |
| Anti-E (DEM1) | Human monoclonal IgM antibody against blood group antigen E | Alba Bioscience |
| Anti-c (H48) | Human monoclonal IgM antibody against blood group antigen c | Alba Bioscience |
| Anti-D (LDM1) | Human monoclonal IgM antibody against blood group antigen D | Alba Bioscience |
| Anti-D (LDM2) | Human monoclonal IgM antibody against blood group antigen D | Alba Bioscience |
| Anti-D (LDM3) | Human monoclonal IgM antibody against blood group antigen D | Alba Bioscience |
| Anti-D (ESD1M) | Human monoclonal IgM antibody against blood group antigen D | Alba Bioscience |
| Anti-D (LDM77/64) | Human monoclonal IgM antibody against blood group antigen D | Alba Bioscience |
| Anti-D (LHM76/58) | Human monoclonal IgG antibody against blood group antigen D | Alba Bioscience |
| Anti-D (LHM76/59) | Human monoclonal IgG antibody against blood group antigen D | Alba Bioscience |
| Anti-D (LHM50/2B) | Human monoclonal IgG antibody against blood group antigen D | Alba Bioscience |
| Anti-D (LHM169/81) | Human monoclonal IgG antibody against blood group antigen D | Alba Bioscience |
| Anti-D (ESD1) | Human monoclonal IgG antibody against blood group antigen D | Alba Bioscience |
| Anti-D (LHM76/55) | Human monoclonal IgG antibody against blood group antigen D | Alba Bioscience |
| Anti-D (LHM77/64) | Human monoclonal IgG antibody against blood | Alba Bioscience |

| | | |
|---|---|---|
| | group antigen D | |
| Anti-D (LHM70/45) | Human monoclonal IgG antibody against blood group antigen D | Alba Bioscience |
| Anti-D (LHM59/19) | Human monoclonal IgG antibody against blood group antigen D | Alba Bioscience |
| Anti-D (LHM169/80) | Human monoclonal IgG antibody against blood group antigen D | Alba Bioscience |
| Anti-D (BRAD3) (9433P) | Human monoclonal IgG antibody against blood group antigen D | National Blood Service |
| Anti-D Polyclonal | Human polyclonal IgG antibody against blood group antigen D | Alba Bioscience |
| Anti-HIV (HIVG1) | Mouse monoclonal IgG antibody against HIV gp140 | Alba Bioscience |
| Anti-HIV (HIVG2) | Mouse monoclonal IgG antibody against HIV gp140 | Alba Bioscience |
| Anti-K (MS56) | Human monoclonal IgM antibody against blood group antigen K | Serologicals |
| Anti-$IgG_3$ (LG3A) | Mouse monoclonal IgG antibody against human $IgG_3$ | Alba Bioscience |
| Anti-human IgG | Rabbit polyclonal anti-human IgG | Alba Bioscience |
| BSA | Bovine Serum Albumin | ID Bio |
| FCS | Foetal Calf Serum | Alba Bioscience |
| Synthetic A antigen | Blood group A trisaccharide conjugated to BSA | Dextra Laboratories |
| Synthetic B antigen | Blood group B trisaccharide conjugated to BSA | Dextra Laboratories |
| Cy3 Anti-Human IgG | Cy3 labelled goat antibody against human IgG | Sigma-Aldrich |
| Cy3 Anti-Human IgM | Cy3 labelled goat antibody against human IgM | Sigma-Aldrich |
| FITC Anti-Mouse IgG | FITC labelled goat antibody against mouse IgG | Sigma-Aldrich |
| FITC Anti-Mouse IgM | FITC labelled goat antibody against mouse IgM | Sigma-Aldrich |
| FITC ConA | FITC labelled Concanavalin A | Sigma-Aldrich |
| FITC Anti-Rh29 | FITC labelled mouse monoclonal IgG antibody against blood group antigen Rh29 | Dr. D.Pepper |
| FITC WGA | FITC labelled wheat germ agglutinin (*Triticum vulgaris*) | Sigma-Aldrich |
| rProtein-L | Recombinant protein-L from *Peptostreptococcus magnus* | Actigen |
| Sheep IgG | Sheep IgG reagent grade | Sigma-Aldrich |
| Goat IgG | Goat IgG reagent grade | Sigma-Aldrich |
| Rabbit IgG | Rabbit IgG reagent grade | Sigma-Aldrich |
| Human Serum | Human serum | Alba Bioscience |
| Bovine Serum | Bovine serum | Sigma-Aldrich |

## 2.2 Antibody Purification Methods

### 2.2.1 IgM Antibody Purification

IgM antibody purification was carried out by either gel filtration or affinity chromatography.

#### 2.2.1.1 IgM Antibody Purification by Gel Filtration

Culture supernatant to be purified was passed through a column that utilises size exclusion to separate the IgM molecule from unwanted molecules in the solution. Smaller molecules are retarded in the porous Sephadex beads and take longer to pass through the column, whereas the large IgM molecules pass directly through and are first to exit the column.

30 g of Sephadex G200 (Amersham Pharmacia, U.K.) was suspended in two litres of PBS pH 7.4 then placed at 4 °C overnight. The buffer was removed and replaced with fresh PBS pH 7.4 and left at 4 °C overnight. This was repeated three times and the supernatant removed.

The suspension was de-gassed for 1.5 hours after then poured into a 2.5 cm x 100.0 cm column. PBS pH 7.4 was pumped into the column at 20 rpm using a Watson Marlow 505S pump with 1 mm bore silicon tubing. This gave a flow rate of 3 ml per minute. As the Sephadex settled with gravity, the excess supernatant was removed and replaced with more suspension until the column was packed.

Column eluate was monitored using an ISCO UA-5 absorbance detector set to 280 nm ($OD_{280}$) with an ISCO T1197038 optical unit. PBS pH 7.4 was pumped through at 20 rpm to establish a baseline on chart recorder paper. The inlet line was placed into 30 ml of sample to be purified, and then this was loaded onto the column via the pump tubing. PBS pH 7.4 was used throughout as running buffer.

When the chart recorder pen showed an increase in $OD_{280}$ the output was collected until the baseline returned to zero. This breakthrough peak containing purified IgM was collected and then measured as in sections 2.3.1.2. and 2.3.2.3. PBS pH 7.4 was pumped through until the entire sample had passed through and the column was determined to be clean (one hour after the chart recorder had returned to baseline).

### 2.2.1.2 IgM Antibody Purification by Affinity Chromatography

Dr. N. O'Looney and C. McKee, of the Scottish National Blood Transfusion Service, carried out this purification and their provision of purified IgM antibodies is gratefully acknowledged. The columns used were 1 ml HiTrap IgM purification HP columns (Amersham Biosciences, U.K.) and the method followed the manufacturers' instructions.

## 2.2.2 IgG Antibody Purification

Antibodies of the IgG class may be purified by the use of affinity chromatography columns. This method is based on the use of an insolubilised ligand (immunoadsorbent), which reacts with the antibody to form an antibody-ligand complex. The column is washed to remove any unbound proteins and other unwanted

components, then treated with a medium that dissociates the antibody-antigen complex and allows collection of the purified antibody. As the structure of $IgG_1$ and $IgG_3$ are different, the immunosorbant is different for each. Some purifications were performed by E. Nelson of Alba Bioscience.

### 2.2.2.1 $IgG_1$ Antibody Purification

7 ml of ProSep Guard Column and 40 ml of ProSep A High Capacity (Millipore, U.K.) were washed in PBS pH 7.4. Washed ProSep Guard Column was poured into a 1.5 cm by 4.0 cm column and the washed ProSep A into a 1.5 cm by 23.0 cm column. 200 ml of ProSep wash buffer was pumped through the column at 60 rpm using a Watson Marlow 505S pump. One mm bore silicon tubing was used and ran at a flow rate of 20 ml/minute until a baseline was established on the chart recorder.

The sample to be purified was loaded (volumes varied depending on quantity available) and the throughput collected. Once the entire sample was loaded 400 ml of ProSep wash buffer was pumped through. Bound antibody was eluted using ProSep elution buffer, and when the chart recorder showed an increase in $OD_{280}$ the eluate was collected. The pH of this breakthrough peak containing purified IgG was adjusted to pH 9.0 using 1M NaOH before being dialysed into PBS pH 7.4 (see 2.2.3). The column was cleaned by running through methylated spirits (70 %) then HCl pH 1.5. Once cleaned, PBS-azide was pumped through and the column was stored at 4 °C in this buffer until required.

### 2.2.2.2 $IgG_3$ Antibody Purification

5 ml of ProSep G High Capacity (Millipore, U.K.) was washed in PBS pH 7.4. The washed ProSep G was then poured into a 1.5 cm by 3 cm column. The purification process was then continued as 2.2.2.1.

## 2.2.3 Dialysis of Purified Antibodies

Dialysis of purified antibodies was performed to transfer antibodies into a storage solution, buffers for use in microarrays, or for use in surface plasmon resonance analysis. This was performed using either dialysis tubing or cellulose membranes. Once dialysed, samples were either stored frozen until required, or used immediately.

### 2.2.3.1 Dialysis of Purified Antibodies Using Dialysis Tubing

Antibody samples purified using ProSep columns were dialysed from the elution buffer into PBS pH 7.4 for storage.

A suitable length of cellulose dialysis tubing, MW cut-off 12,000 (Sigma-Aldrich, D9527) was cut and allowed to soften in PBS pH 7.4 for five minutes. The sample to be dialysed was poured in and both ends sealed. This was then placed into PBS pH 7.4 that was 50 times the volume of the sample and was mixed using a magnetic stirrer and bar, at 4 °C overnight. The following day the sample was removed from the dialysis tubing.

### 2.2.3.2 Dialysis of Purified Antibodies Using Cellulose Membranes

Antibody probes for use in microarrays were dialysed into buffers at different pH. Antibodies for use in surface plasmon resonance analysis were dialysed into sodium acetate, at various pH.

Buffer into which the antibody was to be dialysed was placed in an 85 mm diameter petri dish. The buffer was 100 times the volume of the sample. A cellulose filter membrane disc 0.025 $\mu$m (Millipore, U.K.) was placed onto the buffer with the shiny side facing up. 50 or 100 $\mu$l of sample was pipetted onto the membrane. The sample was allowed to dialyse for one hour at room temperature. The sample was removed from the membrane using a pipette.

### 2.2.4 Concentration of Purified Antibodies

Samples were concentrated using a Filtron Stirred Cell System. A 30,000 molecular weight cut-off Filtron membrane was attached to the bottom of a Filtron vessel. A pressure of one bar was applied to the vessel using nitrogen, and this forced filtrate out the filtrate line while mixing was constant throughout. The process continued until the retentate reached the desired concentration factor.

## 2.3 Quality Control of Antibody Probes and Target Samples

Quality control of antibody probes included both quantitation and functional analysis. The functional analysis was performed by haemagglutination. Quality control of target samples (usually erythrocytes) involved haemagglutination testing,

and the appropriate methods are described here. A summary of all testing performed on the antibody probes used in this project is described in **Table 2.4.**

### 2.3.1 Quantitation of Antibody Probes

#### 2.3.1.1 IgG Antibody Quantitation

IgG antibodies were measured in a Cecil 1000 Series spectrophotometer at $OD_{280}$. 1 ml of material was pipetted into a glass cuvette. The absorbance value was plotted on a standard curve that was prepared with standards at 0.1 mg/ml to 2.0 mg/ml. If the absorbance was above one then the sample was diluted in PBS pH 7.4 and measured. The result was then multiplied by the dilution factor.

#### 2.3.1.2 IgM Antibody Quantitation ELISA

Linda Knowles of Alba Bioscience carried out the IgM antibody quantitation by ELISA. This work is gratefully acknowledged. The procedure involved the use of anti-IgM to the appropriate species being tested, and followed standard laboratory protocols.

### 2.3.2 Haemagglutination Assays

Haemagglutination assays were performed to determine functional pre- and post purification activity of antibodies and to confirm the blood type of samples for testing. Erythrocyte concentrations were all measured in a Sysmex KX-21 Automated Haematology Analyser. Blood typing results were all confirmed using blood typing products from Alba Bioscience (Edinburgh, U.K.), following the manufacturers recommended techniques. The system described in **Table 2.3** was

used to grade haemagglutination reactions, performed in tube tests, according to the level of agglutination present.

**Table 2.3.** Grading system used to determine level of agglutination present (U.K. Blood Transfusion Services, 2002).

| Reaction Grade | Description | Titration Score |
|---|---|---|
| Grade 5 | Cell button remains in one clump or dislodges into a few large clumps, macroscopically visible. | 12 |
| Grade 4 | Cell button dislodges into numerous large clumps, macroscopically visible. | 10 |
| Grade 3 | Cell button dislodges into many small clumps, macroscopically visible. | 8 |
| Grade 2 | Cell button dislodges into finely granular but definite, small clumps, macroscopically visible. | 5 |
| Grade 1 | Cell button dislodges into fine granules, microscopically visible | 3 |
| Grade 0 | Negative result | 0 |

For potency determination, doubling dilutions were prepared in PBS-BSA 2 %, before testing the neat reagent and dilutions by the techniques described in 2.3.2.1 and 2.3.2.3. Cells selected for testing were as described in **Table 2.4**. The titration end point, or potency, was the most dilute antibody dilution that gave a reaction grade 2, as described in **Table 2.3**.

**Table 2.4**. Quality control summary of all probes used (n/a not applicable, NT not tested).

| Probe | Purification method | Titration end point | RBC used in titration | Immuno-diffusion | Conc. mg/ml (OD280) | Conc. mg/ml (Agilent) | μg/ml (ELISA) | Purity % |
|---|---|---|---|---|---|---|---|---|
| Anti-A(B) (ES15) | gel filtration | 2048 | $A_1$ | mouse IgM | n/a | NT | 127 | NT |
| Anti-A(B) (ES15) | affinity column | 4096 | $A_1$ | NT | n/a | NT | 425 | NT |
| Anti-A (ES9) | gel filtration | 1024 | $A_1$ | mouse IgM | n/a | NT | 81 | NT |
| Anti-A (ES9) | affinity column | 64 | $A_1$ | NT | n/a | NT | 15 | NT |
| Anti-A (LA2) | gel filtration | 512 | $A_1$ | mouse IgM | n/a | NT | 54.5 | NT |
| Anti-A (LA2) | affinity column | 4096 | $A_1$ | NT | n/a | NT | 1640 | NT |
| Anti-A (DAM1) | affinity column | 512 | $A_1$ | NT | n/a | NT | 71.3 | NT |
| Anti-B (LB2) | gel filtration | 2048 | B | mouse IgM | n/a | NT | 108 | NT |
| Anti-B (LB2) | affinity column | 4096 | B | NT | n/a | NT | 968 | NT |
| Anti-E (DEM1) | affinity column | 16 | $R_1R_2$ | NT | n/a | NT | 437 | NT |
| Anti-c (H48) | affinity column | 1024 | $R_1R_2$ | NT | n/a | NT | 7.5 | NT |
| Anti-D (LDM1) | gel filtration | 16 | $R_1r$ | NT | n/a | NT | 0 | NT |
| Anti-D (LDM2) | affinity column | 8 | $R_1r$ | NT | n/a | NT | 8.7 | NT |
| Anti-D (LDM3) | gel filtration | 8 | $R_1r$ | NT | NT | NT | 34.8 | NT |
| Anti-D (ESD1M) | affinity column | 8 | $R_1r$ | NT | n/a | NT | NT | NT |
| Anti-D (LDM77/64) | affinity column | 8 | $R_1r$ | NT | n/a | NT | 0 | NT |
| Anti-D (LHM76/58) | affinity column | 65536 | $R_1r$ | NT | 1.00 | 0.871 | NT | 70.2 |
| Anti-D (LHM76/59) | affinity column | 65536 | $R_1r$ | NT | 1.60 | 1.383 | NT | 84.0 |
| Anti-D (LHM50/2B) | affinity column | 2048 | $R_1r$ | NT | 1.77 | 0.9 | NT | 72.2 |
| Anti-D (LHM169/81) | affinity column | 16384 | $R_1r$ | NT | 1.28 | 0.904 | NT | 73.0 |
| Anti-D (ESD1) | affinity column | 8192 | $R_1r$ | human IgG | 1.02 | 0.6 | NT | 67.4 |
| Anti-D (ESD1) sf | affinity column | 16384 | $R_1r$ | NT | 1.00 | NT | NT | NT |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Anti-D (LHM76/55) | affinity column | 65536 | $R_1r$ | NT | 1.8 | 2.184 | NT | 91.2 |
| Anti-D (LHM77/64) | affinity column | 1024 | $R_1r$ | human IgG | 1.07 | 0.992 | NT | 62.7 |
| Anti-D (LHM70/45) | affinity column | 4096 | $R_1r$ | human IgG | 1.38 | 1.606 | NT | 87.8 |
| Anti-D (LHM59/19) | affinity column | 16384 | $R_1r$ | human IgG | 1.07 | 1.553 | NT | 80.8 |
| Anti-D (LHM169/80) | affinity column | 65536 | $R_1r$ | human IgG | 1.09 | 1.317 | NT | 75.1 |
| Anti-D (BRAD3) | as supplied | 32768 | $R_1r$ | NT | 1.0 | NT | NT | Not supplied |
| Anti-D Polyclonal | affinity column | 32 | $R_1r$ | NT | 2.73 | 5.623mg/ml | NT | 80.3 (total IgG) |
| Anti-HIV (HIVG1) | affinity column | NT | NT | mouse IgG | 0.35 | NT | NT | NT |
| Anti-HIV (HIVG2) | affinity column | NT | NT | mouse IgG | 0.34 | NT | NT | NT |
| Anti-K (MS56) | affinity column | 32 | Kk | NT | 1.09 | NT | NT | NT |
| Anti-$IgG_3$ (LG3A) | affinity column | 8192 | IgG sensitised | NT | 1.2 | NT | NT | NT |
| Anti-human IgG | affinity column | 8192 | IgG sensitised | NT | 0.39 | NT | NT | NT |

### 2.3.2.1 IgG Antibody Haemagglutination Assay

80 $\mu$l of IgG antibody was added to a 12 x 75 mm glass tube, then 40 $\mu$l RBC-PBS was added. The tests were mixed thoroughly, then incubated at 37 °C in a water bath for one hour. The tests were washed four times using PBS, using a DiaCent 2 Cell Washing Centrifuge (DiaMed AG, Switzerland). On the last wash the program left a dry button of erythrocytes at the bottom of the tubes. 80 $\mu$l of Anti-Human Globulin reagent (Alba Bioscience) was added to each tube. The tests were mixed thoroughly before being spun at 1000 relative centrifugal force (rcf) for 10 seconds. The tests were read by tipping and rolling the tubes to dislodge the button of cells from the bottom of the tube and then observing for agglutination. Results were graded according to degree of agglutination as described in **Table 2.3**.

### 2.3.2.2 IgM Antibody Haemagglutination Assay

40 $\mu$l of IgM antibody was added to a 12 x 75 mm glass tube, then 40 $\mu$l of RBC-PBS was added. The tubes were shaken and then incubated at room temperature for five minutes. The tests were then centrifuged at 1000 rcf for 10 seconds in a DiaCent 2 Cell Washing Centrifuge (DiaMed AG, Switzerland) The tests were read by tipping and rolling the tubes to dislodge the button of cells from the bottom of the tube and then observing for. Results were graded according to degree of agglutination as described in **Table 2.3**.

### 2.3.2.3 Column Agglutination Testing

#### 2.3.2.3.1 ABO & RhD Blood Group Typing

12.5 $\mu$l of RBC-ID Diluent 2 (5 %) was added to a DiaClon ABO/D + Reverse Grouping card (DiaMed AG, Switzerland) and then spun immediately at 910 rpm for 10 minutes in an ID-24S ID Centrifuge (DiaMed AG, Switzerland). The tests were then read by observing the cards for agglutination.

#### 2.3.2.3.2 IgG Antibody Testing

50 $\mu$l of RBC-ID Diluent 2 (0.8 %) was added to a LISS/Coombs Card (DiaMed AG, Switzerland). 25 $\mu$l of antibody to be tested was added and the card incubated at 37 °C for 15 minutes. The card was spun at 910 rpm for 10 minutes in a 24S-ID Centrifuge (DiaMed AG, Switzerland). The tests were read by observing the cards for agglutination.

### 2.3.3 Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis

Purified antibodies were run on sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) gels to determine level of purification. Novex (U.S.A.) supplied all reagents and equipment unless otherwise stated.

A 4-12 % Bis-Tris Precast Gel Cassette was removed from its pouch and rinsed in water. The tape was removed from the bottom of the cassette and the comb gently removed. The cassette sample wells were rinsed with SDS-PAGE running buffer at least three times and excess was discarded. The cassette was placed in an Xcell II

Mini-Cell E19001 electrophoresis tank and fixed in place using wedges. The tank was filled with SDS-PAGE running buffer.

The sample was diluted to 0.5-2 mg/ml. To 65 $\mu$l of sample, 25 $\mu$l NuPage LDS Sample Buffer (Invitrogen, The Netherlands) and 10 $\mu$l of water were added. The sample was placed in boiling water for 10 minutes. 20 $\mu$l of this sample was added to a well in the gel cassette. 20 $\mu$l of Mark 12 Wide Range Protein Standard was added to one well.

Electrodes were connected as appropriate and the gel was run at 200 V; 120 mA; 24 W for at least 30 minutes or until visible blue lines were within one mm from the end of the gel. The gel was removed from the cassette and immersed in fixing solution (see **Table 2.1**) for 15 minutes with constant mixing. The fixing solution was discarded and replaced with 100 ml of water. This was mixed for 15 minutes and repeated a further three times. The water was discarded and replaced with 50 ml of Gel Code Blue Stain Reagent (Pierce, U.K.) for one hour with constant mixing.

The gel was then dried so it could be retained. The gel was washed twice with water for two minutes. The water was replaced with 35 ml of Gel-Dry Drying Solution (Invitrogen, The Netherlands) and this was mixed for 20 minutes. Two pieces of DryEase Mini Cellophane sheets were soaked in the Gel-Dry Drying Solution for 20 seconds. One sheet was then placed on a DryEase Mini-Gel Dryer Frame and the gel placed in the centre of the sheet. The second sheet was placed on top of this and any wrinkles and bubbles smoothed out. The plastic clamps were pushed onto the four

edges of the frame and the assembly was placed upright on a benchtop. The gel was left for 24 hours until dry and the frame was removed. The gel was placed under light pressure for two days until flat.

The molecular weight of proteins in the sample were assessed by comparing sample bands with bands obtained from the molecular weight markers.

### 2.3.4 Immunodiffusion

Agarose 1 % was prepared and 10 ml poured into each of three petri dishes (85 mm diameter) to give a thin layer. This was allowed to set and using a gel punch, holes were made in the agarose – one large hole in the centre of the plate and six smaller holes near the edges of the plate. 80 $\mu$l of anti-mouse IgM (The Binding Site, U.K.) was added to the centre of one plate, and to each of the other plates 80 $\mu$l of anti-human IgG and 80 $\mu$l of anti-human IgG. 50 $\mu$l of test antibody solutions were added to the smaller surrounding holes on each plate. Six antibodies were tested at the same time.

The plates were allowed to equilibrate for 10 minutes before being placed at 25 °C for 48 hours in a sealed box with an underlayer of wet paper towels. After this time the plates were viewed under a microscope for the presence of immune precipitates, seen as white lines. Where white lines were present the agarose was processed to develop them.

Excess solution was removed from the wells in the plates as necessary and then the agarose was removed from the plate onto a sheet of electrophoresis film (Sigma-Aldrich, U.K.). This was flooded with PBS and then a piece of No.3 Whatman chromatography paper (Sigma-Aldrich, U.K.) was placed on top. On top of this paper was placed eight paper towels followed by a one kg weight. This was pressed for 30 minutes. The agarose plus electrophoresis film was placed into 600 ml of PBS and left overnight. The agarose was then placed on the benchtop and flooded with water and a piece of chromatography paper placed on top. Eight paper towels and a one-kg weight were placed on top of this and left for 30 minutes.

The agarose was dried in an oven (Corning) for 30 minutes and immersed in Crowle's double stain for 30 minutes. The agarose was de-stained for 15 minutes against a 300 ml volume of acetic acid 0.3 % for a total of three washes.

This process demonstrated the type and source of each of the purified antibodies used. If a positive reaction developed it was seen as a blue stained band between the centre well and the test well.

## 2.4 Biotinylation of Antibody

Antibody to be biotinylated was dialysed into bicarbonate buffer pH 9.3. The dialysis procedure described in 2.2.3.1 was followed, except the dialysis tubing was cut and allowed to soften in bicarbonate buffer pH 9.3 for five minutes rather than PBS pH 7.4.

For IgG biotinylation, 10 mg of biotin was added to 0.3 ml of dimethyl sulphoxide (DMSO) and mixed well. For IgM biotinylation 50 mg of biotin was added to 0.3 ml of DMSO. 5 $\mu$l of the appropriate biotin/DMSO was added per one ml of antibody to be biotinylated and allowed to react at 25 °C for one hour. The reaction mixture was then dialysed against PBS pH 7.4. On recovery from dialysis, the mixture was made to final concentration of 5 % glycerol, 1 % Bovine Serum Albumin (ID Bio) and 0.1 % Sodium Azide.

## 2.5 Fluorescent Labelling Procedures

### 2.5.1 Fluorescent Labelling of Antibody/Antigen/Lectin

Fluorescent labelling of antibody, antigen or lectin was carried out using a Cy3 mAb Labelling Kit (Amersham Pharmacia, U.K.). Except the protein to be labelled, all reagents required are included as part of this kit.

Protein to be labelled was prepared to 1 mg/ml (in PBS if required) and 100 $\mu$l was used (0.1 mg). 5 $\mu$l of coupling buffer was then added. The total volume was added to a vial of Cy3 monofunctional dye that was incubated at room temperature for 30 minutes with occasional mixing.

A D-Salt Dextran Desalting Column was prepared by the addition of 3 ml elution buffer, and this was allowed to drain. When the protein/dye mixture was ready it was added to the column. Once the mixture had entered the column, 1.1 ml of elution buffer was added. Another 1.1 ml of elution buffer was added after this. A faster moving pink coloured band developed and was collected when it reached the bottom

of the column. The collected one ml of dye-coupled protein contained 0.1 mg/ml in elution buffer.

### 2.5.2 Fluorescent Labelling of Erythrocytes

0.1 g of fluorescein isothiocyanate (FITC) was dissolved in 10 ml of DMSO in a glass vial, to give a 10 mg/ml FITC/DMSO solution. This was stored at –40 °C until required.

10 ml of a RBC-PBS suspension was placed in a plastic universal containing a circular magnetic stirrer on a magnetic stirrer pad. A pH probe was placed into the solution and the pH was measured throughout. The pH of the solution was adjusted to between pH 8.5 and 9.0 using 1M NaOH. 100 $\mu$l of 10 mg/ml FITC/DMSO solution was added drop by drop into the erythrocyte suspension. The pH was maintained between 8.5 and 9.0. The universal was placed in a water bath at 37 °C for two hours. Cells were recovered by centrifugation at 1000 rpm for five minutes in a Jouan CR312 centrifuge, and the supernatant removed.

Red Cell Wash Solution A 3.2 % (Baxter Healthcare Ltd., U.K.) was diluted 1 in 2 in water to give a hypertonic saline solution at 1.6 % NaCl. 10 ml of this was added to the red cells. The solution was mixed and centrifuged and then the supernatant removed. 10 ml of PBS was added and this was repeated for a total of five washes. On the final wash the red cells were suspended to a haematocrit of 2 % in PBS or Alsevers solution (Alba Bioscience) and BSA (ID Bio, France) added to 2 % final concentration.

### 2.5.3 FACS Analysis of Fluorescently Labelled Erythrocytes

A Becton Dickinson FACScan (fluorescence-activated cell sorter) was used to perform analysis of labelled erythrocyte suspensions. Samples were run through the machine under the supervision of Dr. Shirley Macdonald, SNBTS National Science Laboratory. Her assistance is gratefully acknowledged. Labelled cells were diluted to give approximately 2 x $10^6$ /ml. 10,000 events were counted in the FL1 channel (flow channel one). Events above 10 in this channel were counted as labelled.

## 2.6 Microarray Methods

### 2.6.1 Slide Printing

Slides used to fabricate protein microarray chips were sourced as described in **Table 2.5**. The poly-L-lysine slide preparation followed the method described by the Stanford Microarray Group (cmgm.stanford.edu/pbrown/protocols/1_slides.html). Microarray fabrication was primarily performed with a robotic microarrayer. For some studies described in Chapter 6, a manual microarrayer was used and the methods are described here.

#### 2.6.1.1 Robotic Slide Printing

Probe samples to be spotted onto FAST™ slides (Schleicher & Schuell, Germany) were prepared in Schleicher & Schuell 1X Array Buffer.

Probe samples to be spotted onto all other slides were prepared in PBS. 15 $\mu$l of each probe was pipetted into a 384 well microplate (Genetix Ltd., U.K.). A map of the plate was prepared in Microsoft Excel (Microsoft, U.K.) identifying the contents of

each well. The plate and plate map were passed to the Scottish Centre for Genomic Technology and Informatics, (SCGTI) for printing. Alan Ross of the SCGTI is acknowledged for his expertise in printing microarrays, used in many of the experiments within this work.

**Table 2.5.** Sources of slides used in microarrays.

| Slide Description | Source |
|---|---|
| GAPS (*γ*-aminopropylsilane) | Dr. D. Pepper |
| Gold (BioGold) | Erie Scientific |
| Nitrocellulose (FAST slides) | Schleicher & Schuell |
| P2VP | Dr. D. Pepper |
| P4VP | Dr. D. Pepper |
| PAMAM (poly(amidoamine)) | Dr. D. Pepper |
| pDADMAC(polydiallyldimethylammonium chloride) | Dr. D. Pepper |
| PEG (polyethylene glycol) | Dr. D. Pepper |
| PEI (poly(ethylene isopthalate)) | Dr. D. Pepper |
| Polybrene | Dr. D. Pepper |
| Poly-L-lysine | SCGTI/ Dr. D. Pepper |
| SuperProtein | Arrayit |
| ZetaGrip | Arrayit |

Slides were printed using a BioRobotics MicroGrid II Arrayer. The following sizes of solid pins were used throughout as appropriate; 200 $\mu$m, 400 $\mu$m and 700 $\mu$m. Pins were all supplied by BioRobotics. A print head containing 16 pins, in a 4 x 4 formation, was used throughout. Replicates of each sample were printed on each slide, but this varied depending on the number of probe samples, and will be detailed where appropriate.

Printed slides were allowed to air dry for at least one hour, before being sealed in a bag and placed at 4 °C until required. The MicroGrid II also generates a

QuantArray® (GSI Lumonics, Canada). input file, which was used in interpretation/processing steps. This file gives the location of each spot and the details of the probes printed at that location.

#### 2.6.1.2 Manual Slide Printing

A manual microarrayer from V&P Scientific Inc. was used to manually prepare microarrays. One mm solid steel pins were used. Pins were rinsed in water then in absolute ethanol and dried completely before repeat immersion into probe solution. Probes were printed at least in duplicate. The spacing between spots was facilitated using the grid slots provided on the manual microarrayer.

Printed slides were allowed to air dry for at least one hour, before placing at 4 °C until required. An array map of the slides was self-generated and used in analysis. Slides were subsequently processed using the methods described below.

### 2.6.2 Slide Processing

#### 2.6.2.1 Slide Processing Method A

This process was used for the first protein microarrays that were performed in this work and was based on the method by Haab *et al.* (2001); adjustments and improvements were made on this procedure in subsequent experiments.

Protein microarrays were rinsed briefly in PBST-milk by submerging 10 times, then blocked by placing into a fresh container of PBS-milk. This was placed overnight at 4 °C.

On removal from the blocking solution, the slides were rinsed briefly in PBS three times for at least one minute. The slides were shaken dry and, without allowing the slide to dry after the final wash, a Lifter Slip™ (Erie Scientific) was placed over each array. The protein solution to be added was prepared in PBS-milk and 25 $\mu$l was pipetted under the Lifter Slip™. The slides were placed in a sealed box with an under layer of PBS then placed at 4 °C for two hours.

The slides were briefly submerged into PBS to remove the cover slip and excess protein solution. They were transferred to PBST for 20 minutes with constant mixing. This was followed by two washes in PBS for 10 minutes and two washes in water for 10 minutes. After the final wash the slides were centrifuged to dryness and stored in a dust-free dark place until scanned.

### 2.6.2.2 Slide Processing Method B

This method was adapted from Slide Processing Method A with the following changes:

Microarrays were rinsed briefly in PBST-BSA by submerging 10 times, then placed into a fresh container of PBS-BSA. The protein solution to be added was prepared in PBS-BSA.

### 2.6.2.3 Slide Processing Method C

Protein microarrays were placed in PBS in a glass slide dish at room temperature, and allowed to re-hydrate for 10 minutes. Where glass slide dishes were used the

volume in the dish was 200 ml. The slides were then rinsed briefly in PBST-milk before blocking in a container of PBS-milk.

On removal from the blocking solution, the slides were rinsed briefly in PBS and centrifuged to dryness in an Eppendorf 5810R centrifuge at 1000 rpm for one minute.

A Lifter Slip™ (Erie Scientific) was placed over each array. The protein solution to be added was prepared in PBS-milk and 25 $\mu$l was pipetted under the Lifter Slip™. The slides were placed in a Hybe Chamber (Gene Machines) with a 300 $\mu$l under layer of PBS for each slide. The Hybe Chambers have positions for two slides, each with a reservoir for solution, and the chambers can be completely sealed to avoid evaporation. Once sealed the Hybe Chamber was placed at 4 °C for two hours, or covered with tin foil and left at room temperature if using erythrocytes.

The slides were removed from the Hybe Chambers and briefly submerged into PBS to remove the cover slip and excess protein solution. They were transferred to PBST for 20 minutes with constant mixing. This was followed by two washes in PBS for 10 minutes and two washes in water for 10 minutes. If using erythrocytes, the final washes in water were omitted. After the final wash the slides were centrifuged to dryness and stored in a dust-free dark place until scanning.

When a secondary labelling procedure was required in the microarray experiment, the secondary protein solution was prepared in PBS-milk and 25 $\mu$l was pipetted

under the Lifter Slip™. The remainder of the procedure was as described above, except the incubation period was one hour.

#### 2.6.2.4 Slide Processing Method D

Protein microarrays were placed in PBS in a glass slide dish at room temperature, and allowed to re-hydrate for 10 minutes. The slides were then rinsed briefly in a PBST-milk before being blocked in a container of PBS-milk for one hour at room temperature, with constant mixing.

On removal from the blocking solution, the slides were rinsed briefly in PBS and centrifuged to dryness in an Eppendorf 5810R centrifuge at 1000 rpm for one minute.

A Hybridisation Chamber™ (Schleicher & Schuell) was placed over each array. The protein solution to be added was prepared in the PBS-milk and 450 $\mu$l was pipetted through one of the portholes in the Hybridisation Chamber™. The portholes were sealed with the provided port seals. The slides were placed in a slide box and mixed throughout the incubation period (two hours, reduced to one hour when optimised in Chapter 4) at room temperature.

The Hybridisation Chambers were peeled off and the slides then briefly submerged into PBS to remove excess target solution. They were transferred to PBST for 20 minutes with constant mixing. This was followed by two washes in PBS for 10 minutes and two washes in water for 10 minutes. If using erythrocytes, the final

washes in water were omitted. After the final wash the slides were centrifuged to dryness and stored in a dust-free dark place until scanning.

If a secondary labelling method was to be used, a new hybridisation chamber was used and the process above repeated.

### 2.6.2.5 Slide Processing Method E

This is the final fully optimised procedure and is derived from Slide Processing Method D, with the following adjustments.

Slides were rinsed in PBST-BSA followed by blocking in PBS-BSA. However, no Tween was used at any stage if performing Rhesus grouping (D, c or E). The incubation times were one hour for both primary and secondary incubations (if performed).

### 2.6.2.6 FAST™ Slide Method

This method was adapted from the process specified by the slide manufacturer (Schleicher and Schuell, Germany). Printed protein microarrays were processed as follows. A Hybridisation Chamber (Schleicher and Schuell) was placed over the array and pressed down to form a seal. 500 $\mu$l of 1X wash/block buffer was added through one of the access ports, which were all then sealed. The slides were incubated at room temperature for two hours, ensuring gentle agitation throughout.

The wash/block buffer was removed using a pipette, and was replaced with 500 $\mu$l target protein solution, which had been prepared in 1X wash/block buffer. The slides were then incubated at room temperature for one hour, ensuring gentle agitation throughout.

The protein solution was removed from the slide and the Hybe Chambers were discarded. The slides were then placed in 1X wash/block solution for 10 minutes with constant mixing. This was repeated for a total of three washes. After the final wash the slides were allowed to air dry until the coating on the surface of the slides appeared white. They were then stored in a dust-free dark place until scanning.

### 2.6.3 Slide Scanning

#### 2.6.3.1 Slide Scanning Method A

Slides were scanned in an Affymetrix 428 Array Scanner. Cy3 or Cy5 settings were pre-set, and the Cy3 setting was used to scan FITC where appropriate. The appropriate slide setting was selected for the type of slide to be scanned i.e. glass or nitrocellulose. Photo-multiplier tube (PMT) settings were altered as appropriate. Each slide to be analysed was scanned with at least three different PMT settings, as multiple scanning is recommended by Forster *et al.* (2003). All slide scans were performed at 10 micron pixel size resolution and saved to compact disc as both a bitmap (bmp) and a tagged image file (tif) format.

### 2.6.3.2 Slide Scanning Method B

Slides were scanned in a Packard BioScience (Billerica, MA, USA) ScanArray 5000. Cy3 or Cy5 settings were pre-set, and the Cy3 setting was used to scan FITC where appropriate. PMT and laser power settings were altered as appropriate. Each slide to be analysed was scanned with at least five different settings. Five scans were selected as optimum as described by Forster *et al.* (2003). All slide scans were performed at 10 micron pixel size and saved to compact disc as both bmp and tif formats. This scanner was used latterly as it was automated to batch scan slides.

### 2.6.3.3 Slide Scanning Method C

Slides were scanned in an Genepix Personal 4100A Scanner (Axon Instruments) Settings were pre-set, and the Cy3 setting was used to scan FITC where appropriate. The photo-multiplier tube (PMT) value was altered as appropriate. Each slide to be analysed was scanned at one setting, which was selected by a visual inspection of the scan. All slide scans were performed at 10 micron pixel size and saved to compact disc as both a bmp and a tif.

## 2.6.4 Data Processing Methods

Data was extracted from tif files as described below.

### 2.6.4.1 Data Processing Method A

Numerical data was extracted from the microarray tif files using QuantArray® Microarray Analysis Software (GSI Lumonics, Canada). A protocol for each experiment was prepared using the QuantArray® Protocol Wizard steps. These steps

included setting of the array pattern, grid and spot elasticity, quantitation method and the quantitation parameters. The array pattern was entered using the Pattern Wizard that was used to indicate the location and size of spots in the array. The grid and spot elasticity values were set at 50 throughout. At the quantitation step, the desired quantitation method for each array was selected (histogram, fixed-circle or adaptive) and the location and file name of the QuantArray® input file generated from the MicroGrid II print run was entered. This process gave an average fluorescence intensity value from the centre of each spot and an average background value from the area surrounding each spot.

The QuantArray® program provided a text worksheet that was opened and saved as a Microsoft Excel worksheet.

### 2.6.4.2 Data Processing Method B

Numerical data was extracted from the microarrays using GenePix Pro 4.1 (Axon Instruments). The software controls the scanning, data input and date extraction from the microarray. A text input file was self-generated using microarray column and row positions to determine identity and location of each probe. This was used to generate an array list that was loaded once the microarray grid settings had been set up. Once the grid and the array list had been generated, the data was extracted to a text file. This process gave the median fluorescence intensity value from the centre of each spot and a median background value from the entire background area of the slide.

The program provided a text worksheet that was opened and saved as a Microsoft Excel worksheet.

### 2.6.5 Data Analysis Methods

#### 2.6.5.1 Data Analysis Method

To determine data quality, the data were sorted according to intensity value and a Microsoft Excel line graph was plotted using all intensity values versus all background values. The plotted graph showed if the intensity values for each spot were generally higher than the background values. Data that did not show this were disregarded, and no further analysis performed as the signal to noise ratio was too low.

Data of suitable quality were processed using Microsoft Excel as follows. For each spot the background fluorescence value was subtracted from the fluorescence intensity value. For each slide the signal intensity values from each different scan setting were collated into one worksheet. A scatter plot was prepared using all values for each of the settings set against each other. The shape of the resulting data cloud gave an indication of the scan qualities, and can show if settings were too low, or if settings were too high giving saturated spots. The $R^2$ value was applied to each graph and those that gave a value closest to one demonstrated the best data. One scan from each slide was selected for further data processing.

Once the best data scan had been selected it was processed as follows. Unwanted data were removed from the worksheet to leave only one value per spot on the

microarray (the fluorescence intensity value minus the background fluorescence value for each spot). The negative control values were used to calculate a 'noise' value – the mean plus two standard deviations of the negatives (mean + 2sd). This value represents non-specific binding (NSB). The value for each spot was divided by the mean + 2sd of the negative controls to give a signal-to-noise ratio (S/N). Values over one can be considered significant. The median of the S/N was calculated for the replicate spots of each sample.

Using Microsoft Excel the processed data was analysed as appropriate. Bar charts were used throughout to analyse data. The Y-axis on the bar charts represents the S/N median for the sample.

Where error bars were included, the standard error for each sample was calculated as follows. The standard deviation of the replicates of each sample was calculated (this was performed on S/N ratios or actual fluorescence values). The standard deviation was divided by the square root of the number of replicates of the sample to give the standard error. Error bars were appended using the Microsoft Excel 'Format Data Series' function, or by importing the data into SigmaPlot version 9.0 (Systat Software, U.K.) and indicating the standard error to be used for each sample to give Y error bars. Error bars were added either from replicates on a slide or from replicate slides, and show the level of variability between the data points.

### 2.6.5.2 Normalisation of Data for Slide Comparison

Where comparisons were to be made between slides where a S/N ratio could not be used as a comparator, then the data was normalised. Normalisation helps neutralise non-biological effects (Forster *et al.*, 2003) between microarrays, such as scanner settings and processing differences.

For each signal intensity column, the Microsoft Excel percentile function was used to calculate the 75$^{th}$ percentile value for the entire slide. For slides to be compared the 'best' slide was selected and its percentile value divided by the percentile value of each of the other slides. The resulting ratio for each slide was used as a multiplication factor for all of the spots on the slide.

## 2.7 Blood Typing in Enzyme-Linked Immunosorbent Assay (ELISA) Format

This method was used to test blood typing antibodies with directly and indirectly FITC labelled erythrocytes, to establish if blood typing could be performed in solid-phase using the stated reagents. This uses ELISA principles but the detection was by eye or fluorescence.

The pH of antibodies to be coated onto the ELISA plate was adjusted to 9.0 using 1 M NaOH. 100 $\mu$l of each sample was added in triplicate to the wells of a MaxiSorp ELISA plate (NUNC). ELISA coating buffer was added to several wells to provide background values. The plate was wrapped in cling film and incubated at 37 °C for two hours.

Excess antibody solution was discarded and the plate was washed four times by adding 250 $\mu$l of ELISA wash buffer per well and discarding forcibly. On the last wash the plate was blotted onto paper towels to leave the plate as dry as possible. 250 $\mu$l of ELISA blocking buffer was added per well and the plate was wrapped in cling film and placed at 37 °C for one hour. The plate was washed and dried as before. 100 $\mu$l of a RBC-PBS suspension was added to the appropriate wells. The plate was wrapped in cling film and incubated at 4 °C for one hour. The plate was washed four times as before.

Where directly FITC labelled erythrocytes had been used, the plate was viewed using an inverted microscope for the presence of erythrocytes and then read as below. If unlabelled erythrocytes had been used, 100 $\mu$l of FITC ConA 0.1 mg/ml was added per well. The plate was wrapped in cling film and incubated at 4 °C for one hour. The plate was washed and dried as before and viewed under an inverted microscope before reading for fluorescence as described below.

The plate was read in a Wallac Victor 2 1420 Multilabel Counter using the pre-set setting 'Fluorescein 1.0 seconds'. The results were printed out in a plate format.

## 2.8 Antibody-Antigen Interaction Analysis Using Surface Plasmon Resonance

The Biacore X, Biacore CM5 chip, Biacore X Control Software and BIAevaluation Software (Biacore AB) were used throughout. All data was saved onto compact disc before manipulation of data. The manuals supplied with the Biacore X gave detailed

information on the set up of the machine and experimental options. Where option choices were available in the manuals, these were the methods selected.

A reference flow cell was set up when appropriate, which has been treated in the same way as the active flow cell but had no contact with ligand. Using this, the machine has the facility to perform reference subtraction, where the effects of bulk refractive index changes are corrected together with non-specific binding. Where appropriate, data presented in this work shall be reference subtracted using the reference flow cell values.

### 2.8.1 Preparation of the Biacore-X™

The Biacore-X™ processing unit, PC and printer were switched on and allowed to stabilise for one hour. A Biacore CM5 Sensor Chip was removed from cold storage and the temperature equilibrated to room temperature for at least 30 minutes. The sensor chip was docked and the machine primed with Biacore running buffer. Data collection was initiated by commencing a sensorgram and setting the flow over flow cells 1 and 2 to 5 $\mu$l per minute. This sensorgram was run for at least 30 minutes before any procedure was carried out to allow the sensorgram reading to stabilise.

### 2.8.2 pH Scouting

pH scouting is a procedure to find the pH at which optimal electrostatic binding of the ligand will occur. At this pH the ligand is more likely to be attracted to the sensor surface for covalent binding following chip surface activation.

The ligand (antibody, lectin or synthetic blood group antigen conjugate) was dialysed or diluted into sodium acetate 10 mM (at desired pH). The concentration of ligand was approximately 50 $\mu$g/ml. Dialysis was performed using the method described in 2.2.3.2. pH scouting was performed on an unactivated chip. The sensorgram baseline was set before each injection of ligand. Starting with the highest pH, the ligand solutions were injected over the flow cell for a contact time of 2 minutes at a flow rate of 20 $\mu$l per minute. The pH at which the response was highest was the pH selected for amine coupling.

### 2.8.3 Coupling of Ligand to the Sensor Chip Surface

The coupling of a ligand to the sensor surface involved three main steps: activation of the surface, coupling of the ligand and then deactivation of the surface. This process was performed for each ligand immobilised. The surface of the CM5 chip must first be activated by passing a solution of 1-ethyl-3- (3-dimethylaminopropyl) carbodimide (EDC) and N-hydroxysuccinimide (NHS) over the surface, and a schematic is shown in **Figure 2.1**. The activation time affects the number of sites activated on the chip.

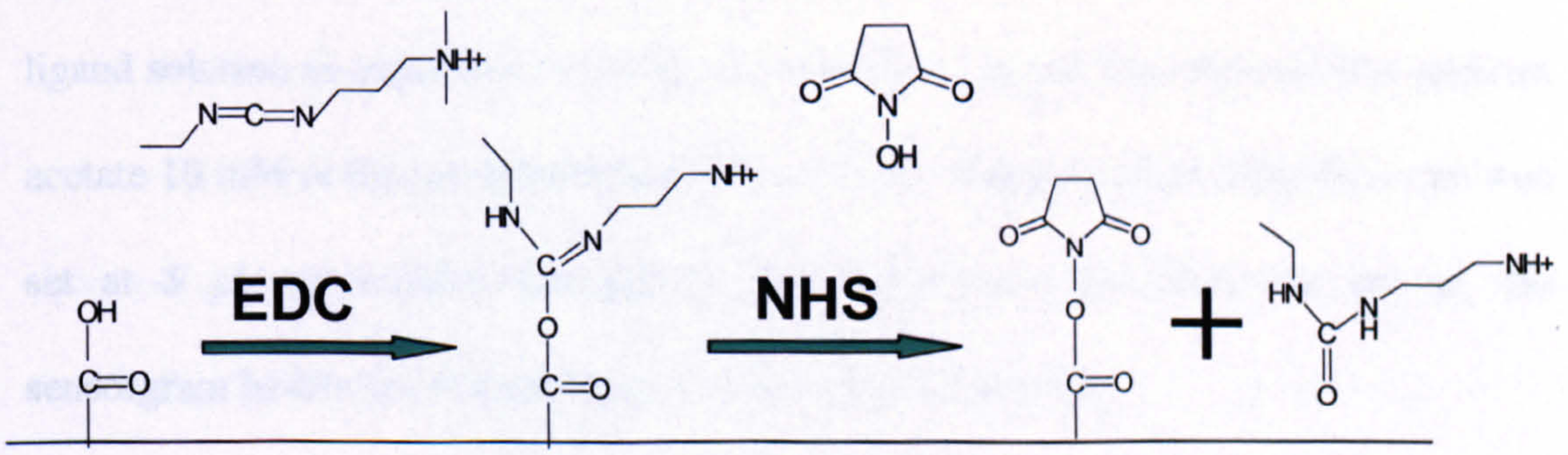



**Figure 2.1**. Schematic showing activation of sensor chip surface by EDC/NHS (dextran chains omitted). (Modified from Biacore Sensor Surface Handbook, 2003).

Once activated, the ligand solution (at appropriate pH) is passed over the sensor surface and the reactive succinimide esters react spontaneously with the amine groups on the ligand, and the antibody or antigen is covalently linked to the chip. The coupling is demonstrated in **Figure 2.2**. It is important that the ligand has been purified, as impurities may be immobilised and affect the binding capacity of the surface.

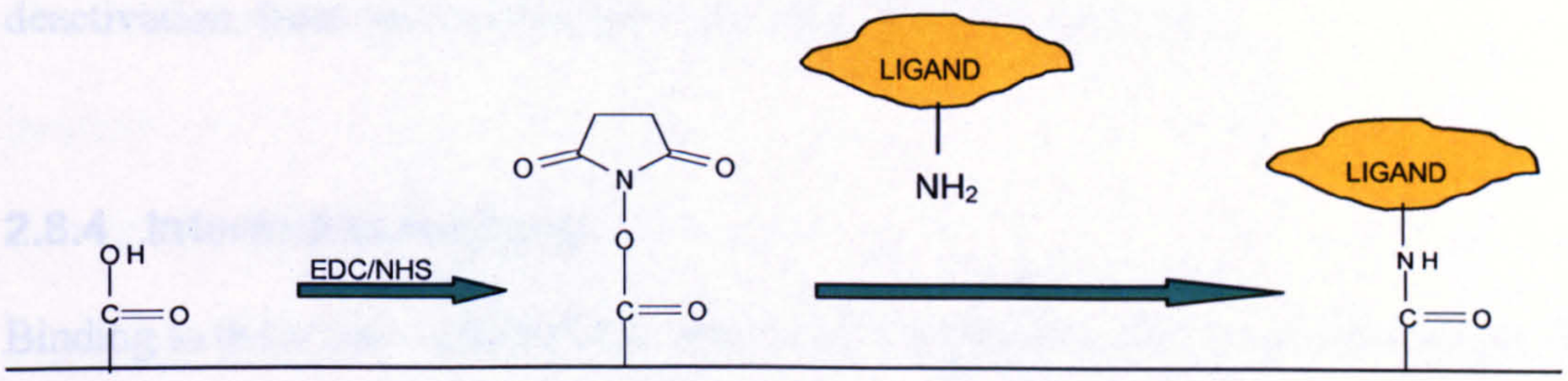



**Figure 2.2**. Schematic showing amine coupling of ligand to the activated surface. (Modified from Biacore Sensor Surface Handbook, 2003).

Ethanolamine is passed over the sensor surface to deactivate uncoupled active esters. This will bind to any remaining reactive groups on the sensor chip surface. This stage is vital to prevent non-specific binding of analyte to the sensor surface.

Ligand was covalently immobilised on the sensor chip surface as follows. 100 $\mu$l of ligand solution at concentration of approximately 50 $\mu$g/ml was dialysed into sodium acetate 10 mM at the pH determined in the pH scouting procedure. The flow rate was set at 5 $\mu$l per minute throughout. The sensorgram baseline was set on the sensorgram before the activation procedure was performed.

The surface of the chip was activated by injection of NHS/EDC over flow cells 1 and 2, for a contact time of 10 minutes. The coupling of ligand was performed by the injection of ligand solution (at desired pH) over flow cell 2 only, for a contact time of 10 minutes. The surface of the chip was deactivated by the injection of ethanolamine over both flow cells 1 and 2 for a contact time of 7 minutes.

The level of immobilisation of ligand was recorded as the response after deactivation, from the sensorgram of flow cell 2 minus flow cell 1.

### 2.8.4 Interaction Analysis

Binding to the sensor chip surface takes place during the injection of the analyte. The change in refractive index (RI) indicated on the sensorgram by a change in RU, demonstrates some change at the sensor chip surface. An increase in the RU means molecules are binding, a decrease means they are dissociating. The injection of sample environments that differ greatly from the running buffer will cause a bulk shift in the RI. An RI shift is demonstrated by a square pulse in the sensorgram and a shift in the RU. To prevent this the analyte is diluted in running buffer before injection.

This procedure, and the initiation of a sensorgram, enables the monitoring of real-time molecular interactions of ligand and analyte on the chip sensor surface. To enable the re-use of the coupled sensor chip, the surface must be regenerated. This is the removal of analyte without the removal of ligand. Regeneration conditions were optimised during this study for each analyte. Regeneration can be achieved by

denaturing the analyte or disrupting the structure. When establishing regeneration conditions, the analyte must be injected over the sensor surface to ensure that the ligand activity has not been affected. If regeneration is not complete then this will affect the binding capacity of the chip. Regeneration is satisfactory if the sensorgram returns to within 5% of the baseline and when the repeat binding of analyte is within 10% of the initial response (Biacore.com).

The analyte (antibody, erythrocyte or synthetic blood group antigen conjugate) was prepared in Biacore running buffer or Biacore running buffer-Mn/Ca (if using Concanavalin A). The analyte was injected over flow cells 1 and 2 for a contact time of 10 minutes at a flow rate of 5 $\mu$l per minute. The baseline was set at the start of the injection. The Biacore normal wash procedure, as indicated in the operation manual, was carried out at the end of the injection.

The surface was regenerated at the end of each injection of analyte and before the next injection. Regeneration was performed using one-minute pulses of 1 mM NaOH. This was injected over both flow cells until the response in sensorgram flow cell 2 minus 1 was within 5 % of the baseline response. The level of binding was recorded as the response from flow cell 2 minus 1, at the end of the injection.

In this work it was found that the bound ligands were not stable after storage following approximately seven day storage (data not shown), therefore any experimental work was performed within two days of the coupling of the ligand.

### 2.8.5 Evaluation of Biacore Data

BIAevaluation software was used to manipulate the sensorgrams. Report point data from sensorgrams were interpreted in tabular form or using Microsoft Excel.

## 2.9 Preparation of Erythrocyte Membrane Protein Fragments

A ProteoExtract™ Native Membrane Protein Extraction Kit (CalbioChem) was used to prepare native membrane protein fragments from erythrocytes. All reagents used were supplied as part of the kit, except the PBS and cell samples. This kit extracts integral and membrane-associated proteins in a non-denatured state based on their association with cellular membranes. A bench top Eppendorf 5415R centrifuge was used throughout this process at the settings indicated.

Erythrocytes were washed in PBS at least four times, then an aliquot containing approximately 1.25 x $10^7$ erythrocytes was processed in an Eppendorf tube. The cells were spun down at 1000 g for one minute and excess supernatant removed. 7.5 $\mu$l of Protease Inhibitor Cocktail was added to the wall of the tube followed by 1.5 ml of ice cold Extraction Buffer I. The pellet was resuspended and mixed gently using a pipette. The tube was incubated at 4 °C for 10 minutes in a rotary shaker. The tube was then centrifuged at 16,000 g at 4 °C for 15 minutes. Following this the supernatant was removed using a pipette, and discarded.

A further 5 $\mu$l of Protease Inhibitor Cocktail was added to the side of the tube followed by 1.0 ml of ice-cold Extraction Buffer II. The pellet was resuspended and mixed gently using a pipette. The tube was incubated at 4 °C for 30 minutes in a

rotary shaker. The tube was then centrifuged at 16,000 g at 4 °C for 15 minutes. The supernatant containing the membrane proteins from the erythrocytes was then collected.

The resultant aliquot contained the protein membranes from 1.25 x $10^7$ erythrocytes, in a volume of approximately 0.9 ml. This was equivalent to protein membranes from 1.4 x $10^4$ erythrocytes/$\mu$l.

To prepare a more concentrated suspension of erythrocyte membrane protein fragments, less Protease Inhibitor Cocktail and Extraction Buffer II were added, in proportion. The volumes used were 1 $\mu$l of Protease Inhibitor Cocktail, followed by 0.2 ml of ice-cold Extraction Buffer II. This contained the protein membranes from 1.25 x $10^7$ erythrocytes, in a volume of approximately 0.2 ml. This was equivalent to protein membranes from 6.25 x $10^4$ erythrocytes/$\mu$l. Aliquots were frozen at –20 °C and thawed when required.

# CHAPTER 3

# DEVELOPMENT AND EVALUATION OF PROTEIN MICROARRAYS FOR THE STUDY OF PROTEIN-LIGAND INTERACTIONS

# 3. DEVELOPMENT AND EVALUATION OF PROTEIN MICROARRAYS FOR THE STUDY OF PROTEIN-LIGAND INTERACTIONS

## 3.1 Introduction and Chapter Aims

Various design options for microarray experimentation were described in Chapter 1 (1.4.2), as was blood testing for transfusion purposes (1.3). In order to examine the interactions of blood group antigens and antibodies, it was necessary to first investigate the antibody-based protein microarray platform and then optimise for simple sets of related proteins and ligands. Protein microarrays for this purpose are not standardised systems available ready-for-use, but research and potentially diagnostic tools in development.

The aims of this initial stage of the project were:

- To determine the ability to fabricate protein microarrays using available equipment and using publications detailing similar type studies, then to probe the microarrays with anti-species detection/target solutions to determine if probes are retained on the slides.
- To optimise protein microarray procedures such as blocking, washing, incubation conditions, detection methods, data extraction and data processing methods, to enable the study of probe-ligand interactions.
- To evaluate different slide surface chemistries and select options for the next stage of the project (blood typing microarrays).
- Amalgamate all findings and process optimisations to provide a basis for a multi-parameter blood typing microarray platform.

## 3.2 Determination of Conditions for Microarray Fabrication

As mentioned in Chapter 1, MacBeath and Schreiber (2000) and Haab *et al.* (2001) had described the use of similar substrates and techniques used in DNA arrays for the preparation of protein microarrays. In order to apply and develop protein microarray procedures for the new application described in this thesis, experiments were devised to investigate if the work of Haab *et al.* could be repeated in our settings. For this purpose, a variety of protein-ligand sets were used, and processed using Haab *et al.* (2001) as a reference point. Probes were then detected using anti-species antibodies and the interactions studied.

Serum proteins, polyclonal and monoclonal antibodies were used throughout this chapter as probes, and fluorescently labelled anti-species antibodies were used for detection (target antibodies). Serum proteins were purchased and used as supplied, or diluted. Antibodies of the IgG class were purified by the use of affinity chromatography columns. IgM antibodies were purified using the methods of gel filtration and affinity chromatography. Protein probes prepared in-house were assessed by various methods for activity, quantitation, and purity as described in Chapter 2.

As discussed in Chapter 1 (1.4.2.2), the choice of probes for any microarray experiment must be considered carefully. Protein probes must include proteins of interest along with suitable controls for subsequent analysis. The proteins printed as probes in this protein microarray experiment are detailed in **Table 3.1.** Bovine serum albumin (BSA) and foetal calf serum (FCS) were included as negative controls.

**Table 3.1.** Protein probes spotted in protein microarray experiment in 3.2, and concentration (conc.) in $\mu$g/ml.

| Protein identification | Protein/Ab type | Conc. DIL1 | Conc. DIL2 | Conc. DIL3 | Conc. DIL4 | Conc. DIL5 | Conc. DIL6 |
|---|---|---|---|---|---|---|---|
| BSA | neg. control | 300 | 200 | 100 | 75 | 50 | 25 |
| ES15 | mouse IgM | 127 | 85 | 42 | 32 | 21 | 11 |
| ES9 | mouse IgM | 81 | 54 | 27 | 20 | 14 | 7 |
| ESD1 | human IgG | 300 | 200 | 100 | 75 | 50 | 25 |
| FCS | neg. control | 300 | 200 | 100 | 75 | 50 | 25 |
| HIVG1 | mouse IgG | 300 | 200 | 100 | 75 | 50 | 25 |
| HIVG2 | mouse IgG | 300 | 200 | 100 | 75 | 50 | 25 |
| LA2 | mouse IgM | 55 | 37 | 18 | 14 | 9 | 5 |
| LB2 | mouse IgM | 108 | 72 | 36 | 27 | 18 | 9 |
| LDM3 | human IgM | 6.4 | 3.2 | 1.6 | 0.8 | 0.4 | 0.2 |
| LHM169/80 | human IgG | 300 | 200 | 100 | 75 | 50 | 25 |
| LHM59/19 | human IgG | 300 | 200 | 100 | 75 | 50 | 25 |
| LHM70/45 | human IgG | 300 | 200 | 100 | 75 | 50 | 25 |
| LHM76/55 | human IgG | 300 | 200 | 100 | 75 | 50 | 25 |
| LHM77/64 | human IgG | 300 | 200 | 100 | 75 | 50 | 25 |

The target proteins, or those used for detection of bound probes, in this experiment were fluorescently labelled anti-species antibodies, and are described in **Table 3.2.**

**Table 3.2.** Target proteins used in protein microarray experiment in 3.2, and concentration.

| Target protein identification | Concentration used ($\mu$g/ml) |
|---|---|
| Cy3 Anti-Human IgG | 33.33 |
| Cy3 Anti-Human IgM | 5.00 |
| FITC Anti-Mouse IgG | 31.25 |
| FITC Anti-Mouse IgM | 7.81 |

Protein microarrays mainly follow the basic principles of standard immunoassays, as described in Chapter 1 (see **Figure 1.8**). In order to describe the process followed in this and subsequent experiments, and to facilitate the reading and interpretation of data, the basic parameters used for each experiment are described as follows:

slide type, number of replicate slides, pin size, probes, on-slide probe replicate spots, slide processing method (SPM), blocker used, target and label, target solution volume, incubation time, static/mixing, scanning method

For instance, using this experiment as an example, the above parameters are summarised in figure legends as follows:

Slide type poly-L-lysine, slide reps 1, pins 700 $\mu$m, probes Table 3.1, probe reps 2, SPM A; blocker PBS-milk, target/volume: Cy3 anti-hIgG/25 $\mu$l, incubation 120 min, static, scanning method A.

Once processed the slides were scanned. Pseudo colour images from the scans are presented in **Figure 3.1** and **Figure 3.2.** The colour spectrum used refers to the intensity of the signal, where black/blue are low fluorescence and red/orange/white are high. The figures show some artefacts on the microarrays, which can complicate analysis if not considered. Examples of 'black holes' are shown in **Figure 3.1a.** Black holes can be a result of non-optimised blocking (u-vision-biotech.com) as the surrounding background to the spot has a higher fluorescence value than the actual spot. The fluorescence present may be autofluorescence from residual buffer, or dust due to poor processing procedures, or that the actual fluorescent antibody has bound non-specifically to the chip.

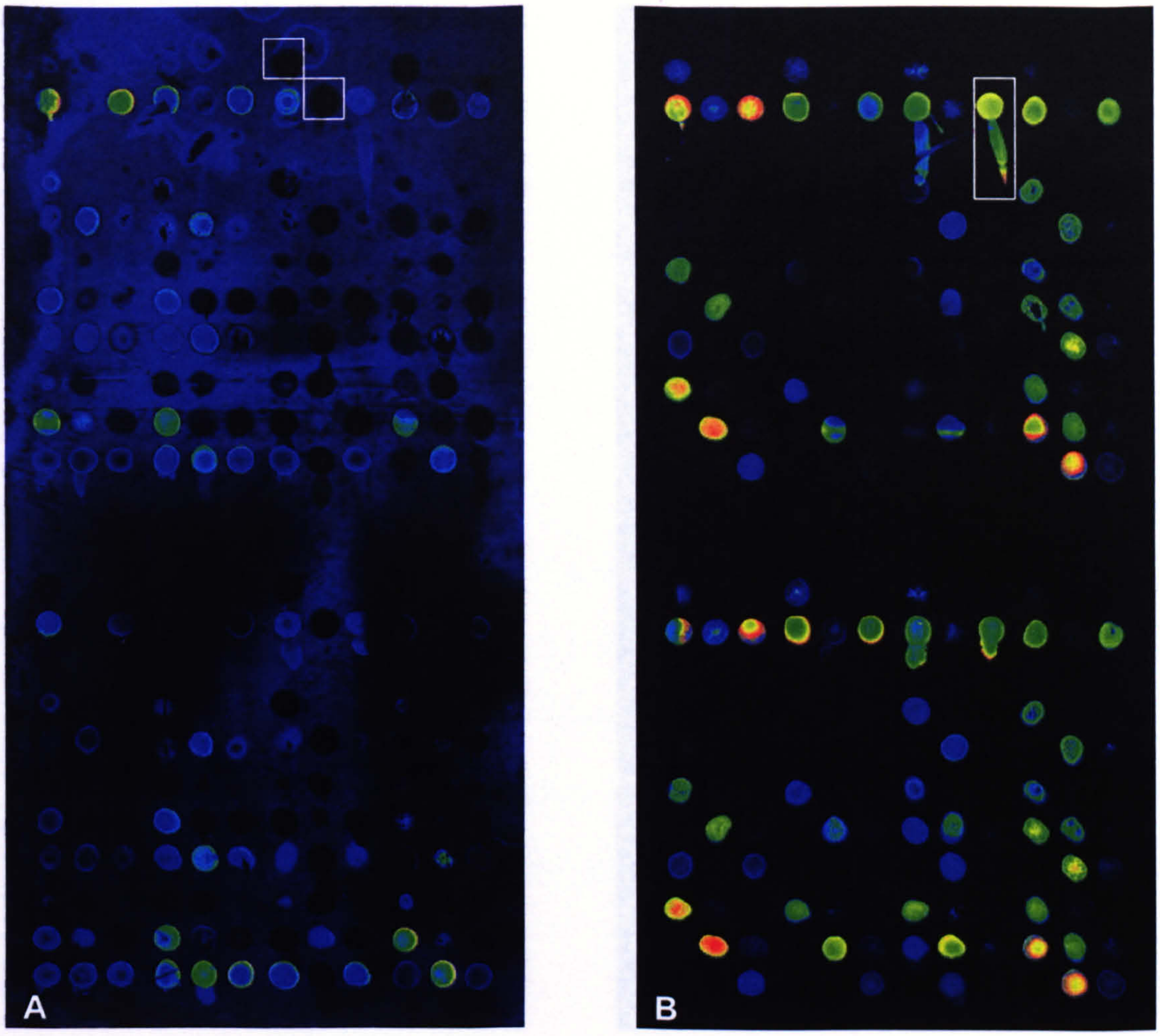



**Figure 3.1.** Pseudo-colour images (blue to white scale) from protein microarray experiment 3.2. The PMT values used for these scans were different (3.1a PMT was 35 and 3.1b PMT was 25). Actual spot size is approximately one mm in diameter.

Fig A. The boxed areas show examples of black holes. Slide type poly-L-lysine, slide reps 1, pins 700 $\mu$m, probes Table 3.1, probe reps 2, SPM A; blocker PBS-milk, target/volume: Cy3 anti-hIgM/25 $\mu$l, incubation 120 min, static, scanning method A.

Fig B. The outlined area shows an example of 'comet-tailing'. Slide type poly-L-lysine, slide reps 1, pins 700 $\mu$m, probes Table 3.1, probe reps 2, SPM A; blocker PBS-milk, target/volume: Cy3 anti-hIgG/25 $\mu$l, incubation 120 min, static, scanning method A.

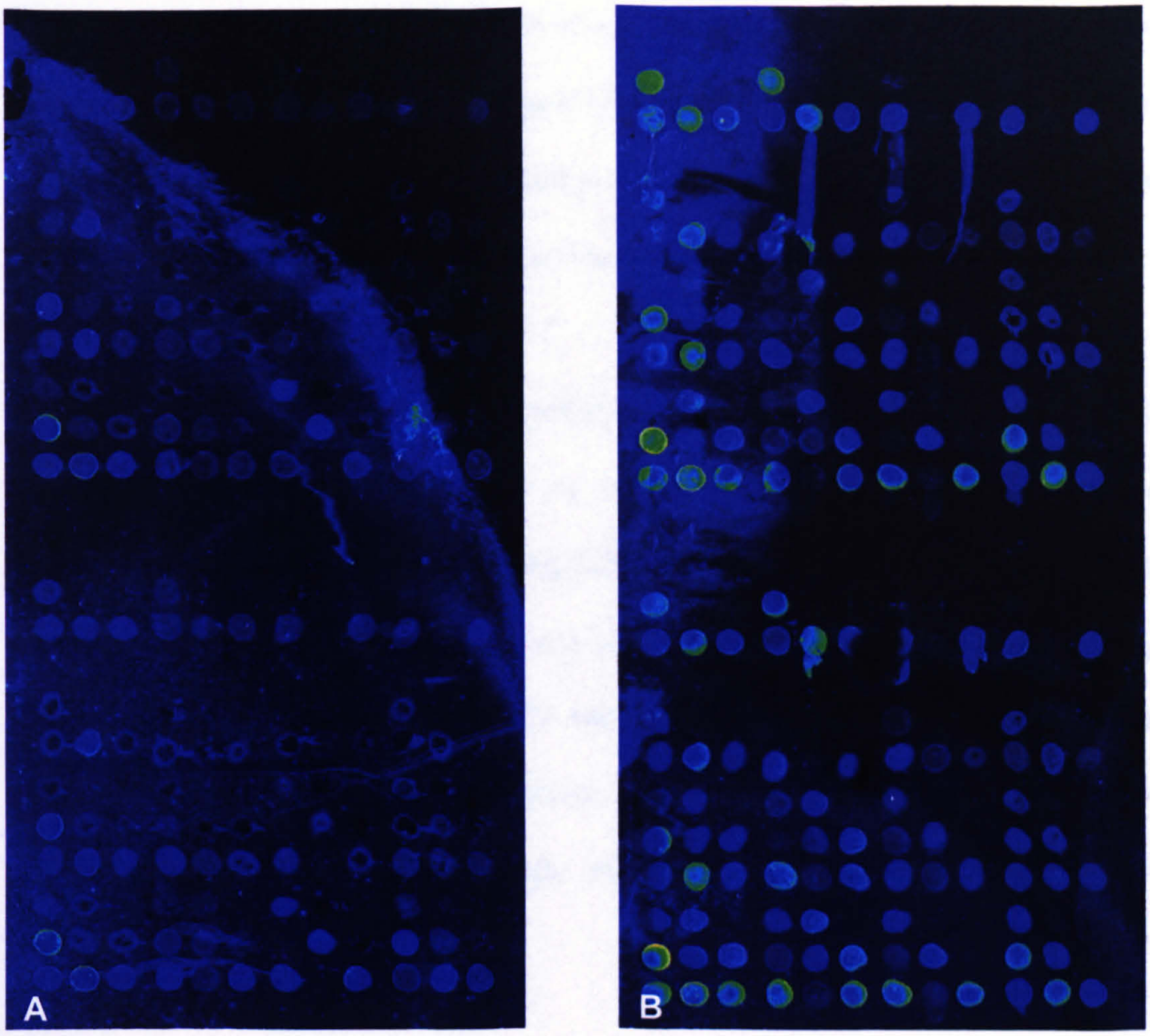



**Figure 3.2.** Pseudo-colour images (blue to white scale) from protein microarray experiment 3.2.
Fig A. Slide type poly-L-lysine, slide reps 1, pins 700 $\mu$m, probes Table 3.1, probe reps 2, SPM A; blocker PBS-milk, target/volume: FITC anti-mIgM/25 $\mu$l, incubation 120 min, static, scanning method A.
Fig B. Slide type poly-L-lysine, slide reps 1, pins 700 $\mu$m, probes Table 3.1, probe reps 2, SPM A; blocker PBS-milk, target/volume: FITC anti-mIgG/25 $\mu$l, incubation 120 min, static, scanning method A.

It is also considered that an increase in protein concentration of a target solution can result in an increase in non-specific background resulting from non-specific binding (NSB) of target proteins (Haab *et al.*, 2001). The black hole effect can present itself in the data analysis as the subtraction of the background from the spot fluorescence can lead to a negative fluorescence value for the spot. An improvement to blocking procedures was later investigated (section 3.3) in an attempt to eliminate or reduce

this effect. In **Figure 3.1b** an improved image is seen as the level of fluorescence to background is much better than in **Figure 3.1a.** Due to the highly specific nature and low non- specific binding, this image was acquired using lower photo-multiplier tube (PMT) settings, which can result in darker background.

However, this image **(Figure 3.1b)** also demonstrates another area for improvement. The highlighted area gives an example of 'comet-tailing'. A more extreme example of comet tailing can result in the lifting-off of the spot itself. This can arise from blocking and, it could be speculated, from overload of sample probes. This smearing effect was reported by Seong (2002) and was found when high probe protein concentrations were used. This phenomenon can also result in negative fluorescence values, as the black hole area usually shows lower values than even negative controls.

The effect of solution drying underneath the cover slip can be seen in **Figure 3.2a** (u-vision-biotech.com), demonstrated by the large amount of background shown in blue. Some spots also show the 'doughnut' effect, where there is circular fluorescence with an area of lower fluorescence in the centre. This can be the result of the pin quality or from lack of humidity during slide drying (u-vision-biotech.com). There is also some variation in spot size.

The effects of some of the phenomenon mentioned above can were overcome. Using statistics, the data can be handled to compensate for background and get the most from the data. Ideally the microarray should have clean and consistent spots and

background, and measures should be taken to minimise unwanted artefacts or unsuitable spots.

The data from the scans shown in **Figures 3.1** and **3.2** was extracted using Data Processing Method A and then manipulated using the Data Analysis Method. These methods will be used throughout this chapter.

Values from the BSA and FCS spots were used as negative controls to calculate noise. The results are presented as bar graphs in **Figures 3.3a-m.** This standard format of data presentation is used throughout this thesis. The y-axis shows the signal to noise ratio (S/N) calculated from the fluorescence values from the probe antibody spot divided by the mean plus two standard deviations of the background noise probes. The x-axis will usually display the probe antibody identification details. Here, the interactions of all four target antibodies with one probe antibody are displayed in one figure. No error bars are used as samples were printed in duplicate.

In order to show the reactivity of the probe mouse IgM antibodies with the fluorescently labelled target proteins the results are compiled in **Figures 3.3a-d.** While the anti-mouse IgM gives low S/N values, the anti-mouse IgG and anti-human IgM show cross-reactivity. Anti-A LA2 also shows reactivity with anti-human IgG.

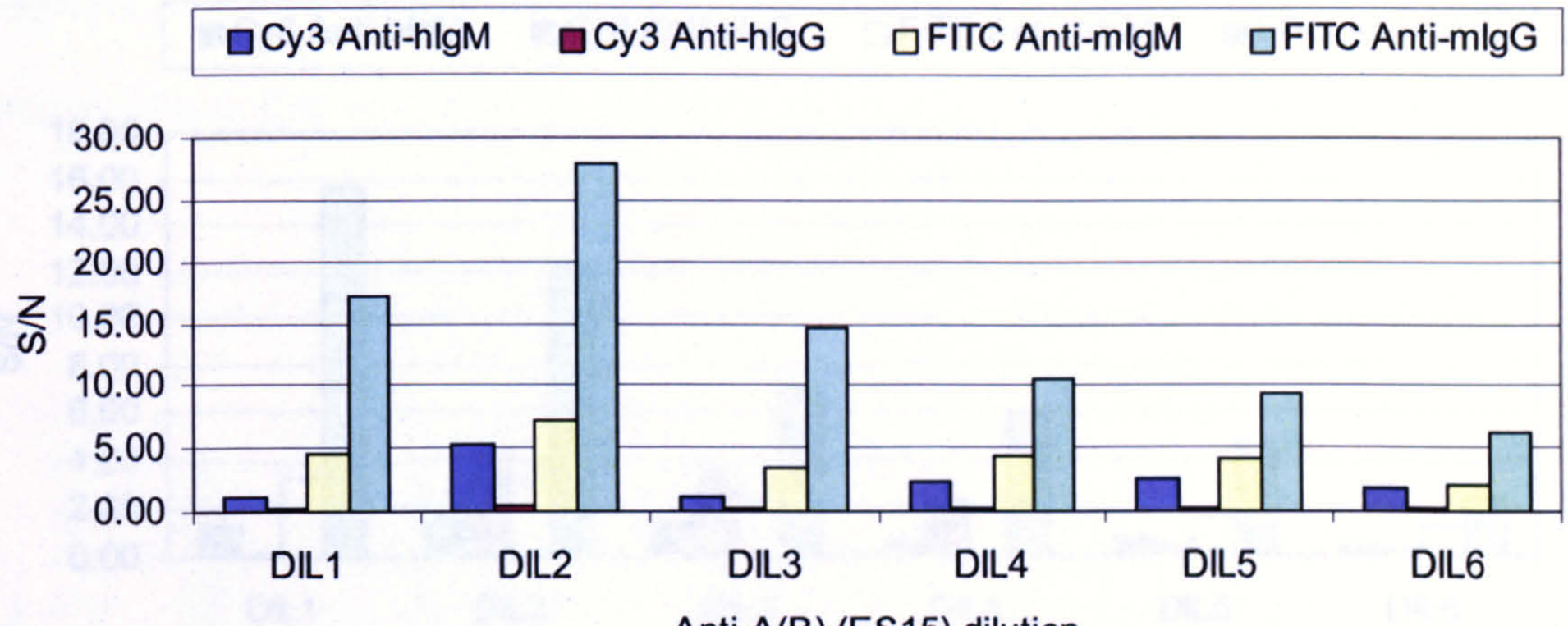



**Figure 3.3a.** Reactions of probe Anti-A(B) ES15 mouse IgM, at various dilutions, with target anti-species antibodies. Slide type poly-L-lysine, slide reps 1, pins 700 $\mu$m, probes Table 3.1, probe reps 2, SPM A; blocker PBS-milk, target/volume: various/25 $\mu$l, incubation 120 min, static, scanning method A.

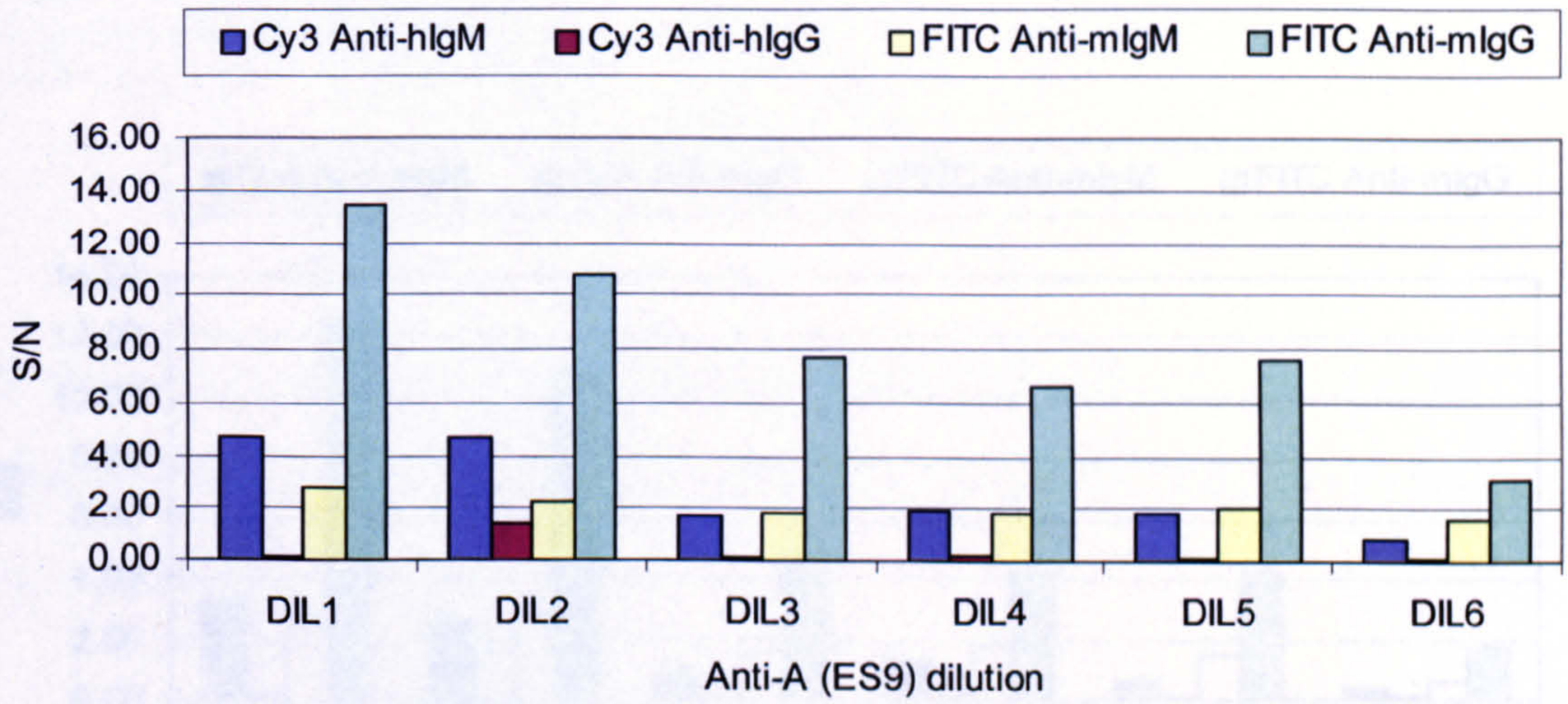



**Figure 3.3b.** Reactions of probe Anti-A ES9 mouse IgM at various dilutions, with target anti-species antibodies. Slide type poly-L-lysine, slide reps 1, pins 700 $\mu$m, probes Table 3.1, probe reps 2, SPM A; blocker PBS-milk, target/volume: various/25 $\mu$l, incubation 120 min, static, scanning method A.

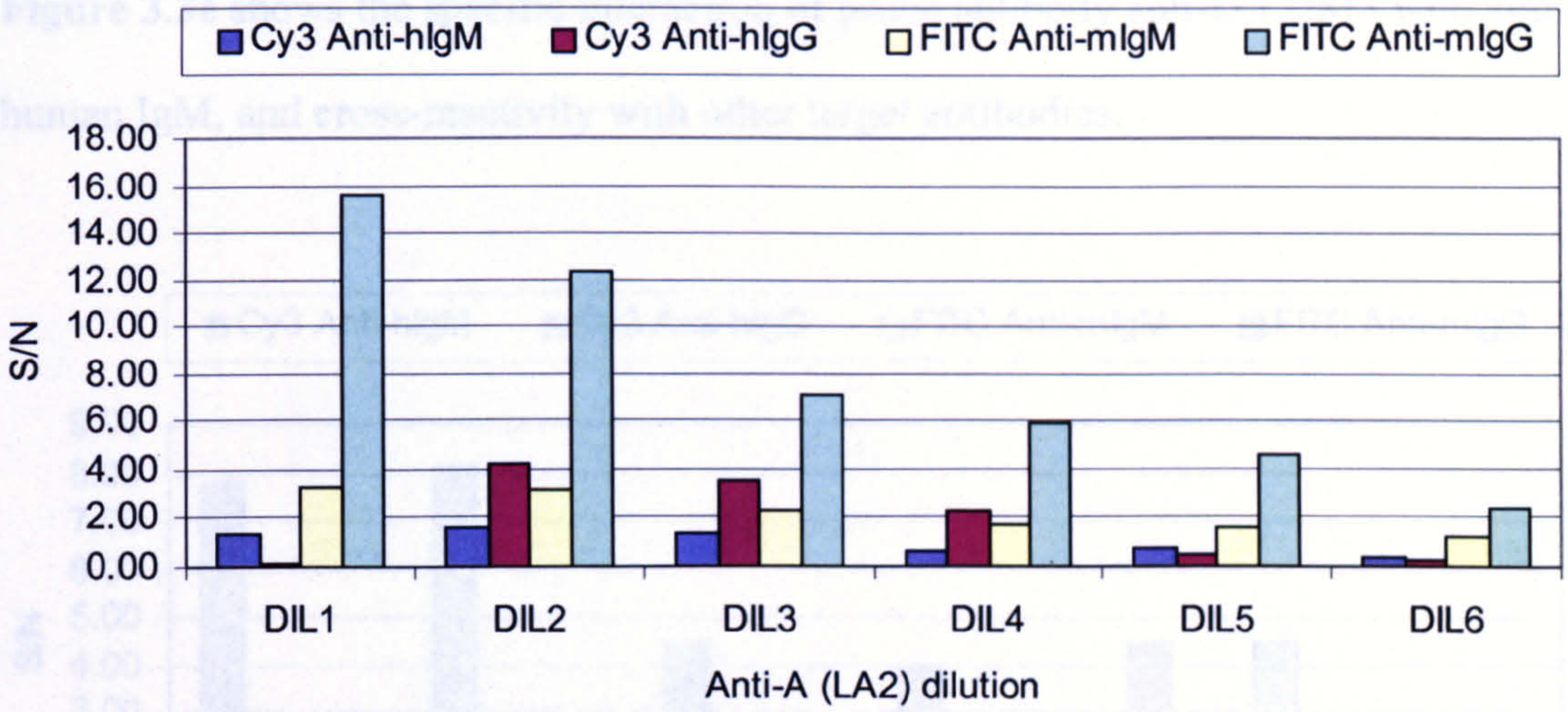



**Figure 3.3c**. Reactions of probe anti-A LA2 mouse IgM at various dilutions, with target anti-species antibodies. Slide type poly-L-lysine, slide reps 1, pins 700 $\mu$m, probes Table 3.1, probe reps 2, SPM A; blocker PBS-milk, target/volume: various/25 $\mu$l, incubation 120 min, static, scanning method A.

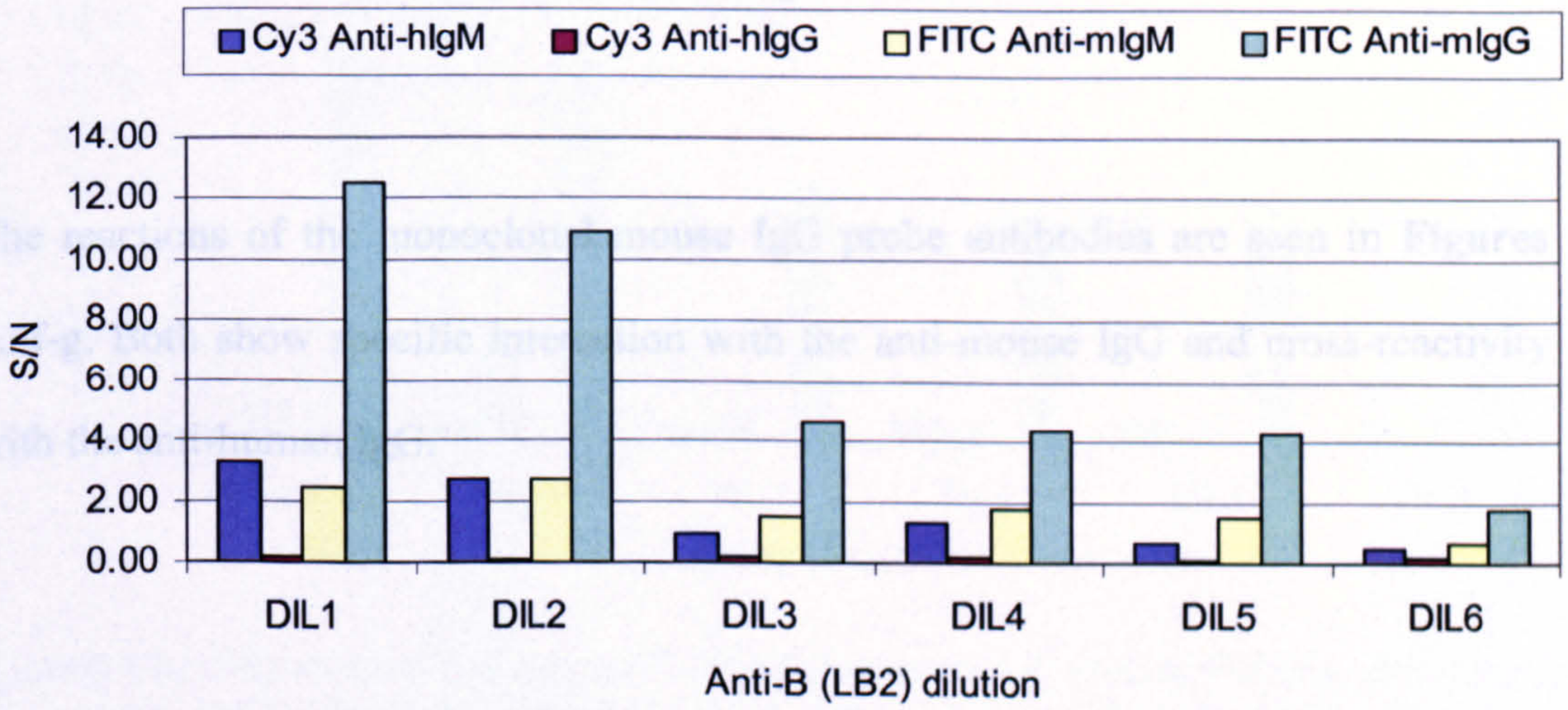



**Figure 3.3d.** Reactions of probe Anti-B (LB2) mouse IgM at various dilutions, with target anti-species antibodies. Slide type poly-L-lysine, slide reps 1, pins 700 $\mu$m, probes Table 3.1, probe reps 2, SPM A; blocker PBS-milk, target/volume: various/25 $\mu$l, incubation 120 min, static, scanning method A.

**Figure 3.3e** shows the specific interaction of probe antibody anti-D LDM3 with anti-human IgM, and cross-reactivity with other target antibodies.

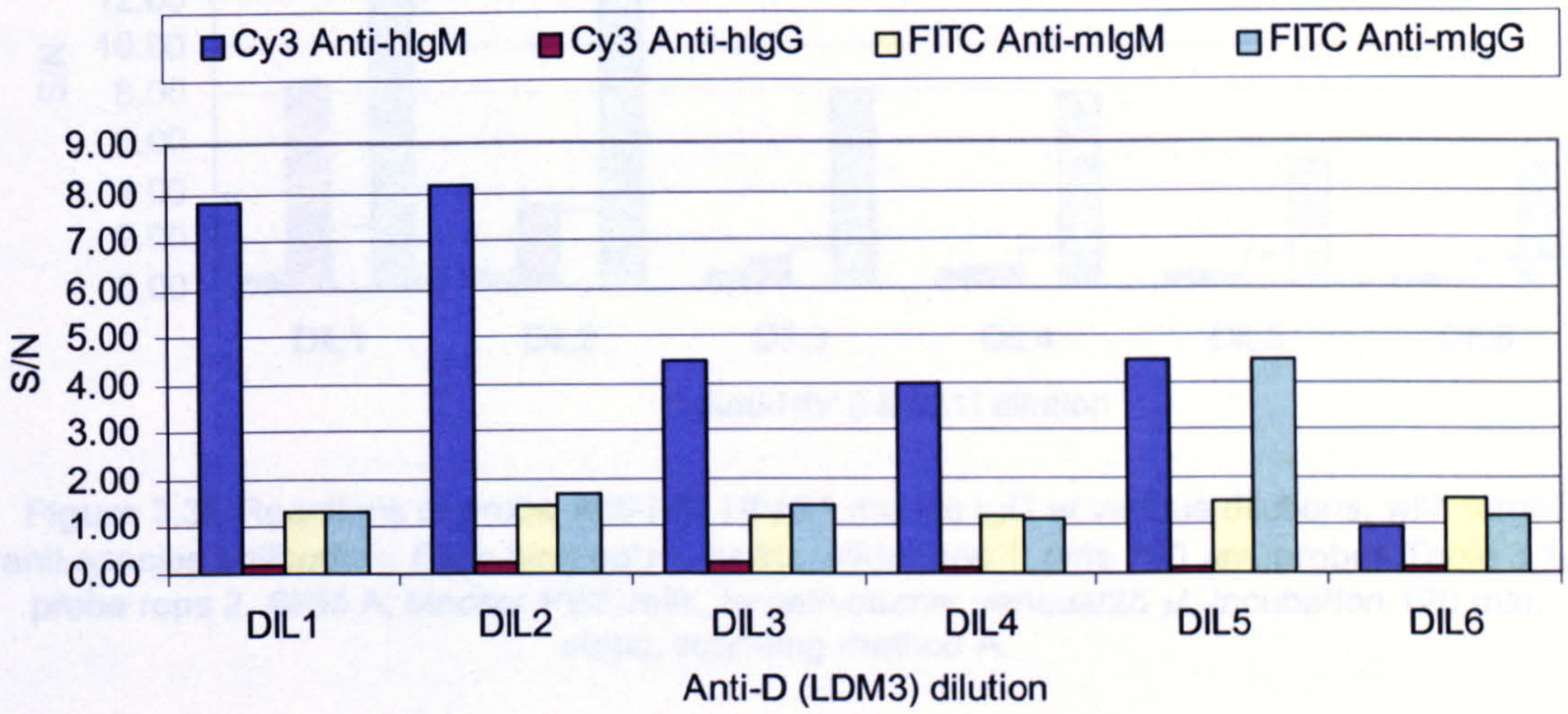



**Figure 3.3e.** Reactions of probe Anti-D LDM3 human IgM at various dilutions, with target anti-species antibodies. Slide type poly-L-lysine, slide reps 1, pins 700 $\mu$m, probes Table 3.1, probe reps 2, SPM A; blocker PBS-milk, target/volume: various/25 $\mu$l, incubation 120 min, static, scanning method A.

The reactions of the monoclonal mouse IgG probe antibodies are seen in **Figures 3.3f-g**. Both show specific interaction with the anti-mouse IgG and cross-reactivity with the anti-human IgG.

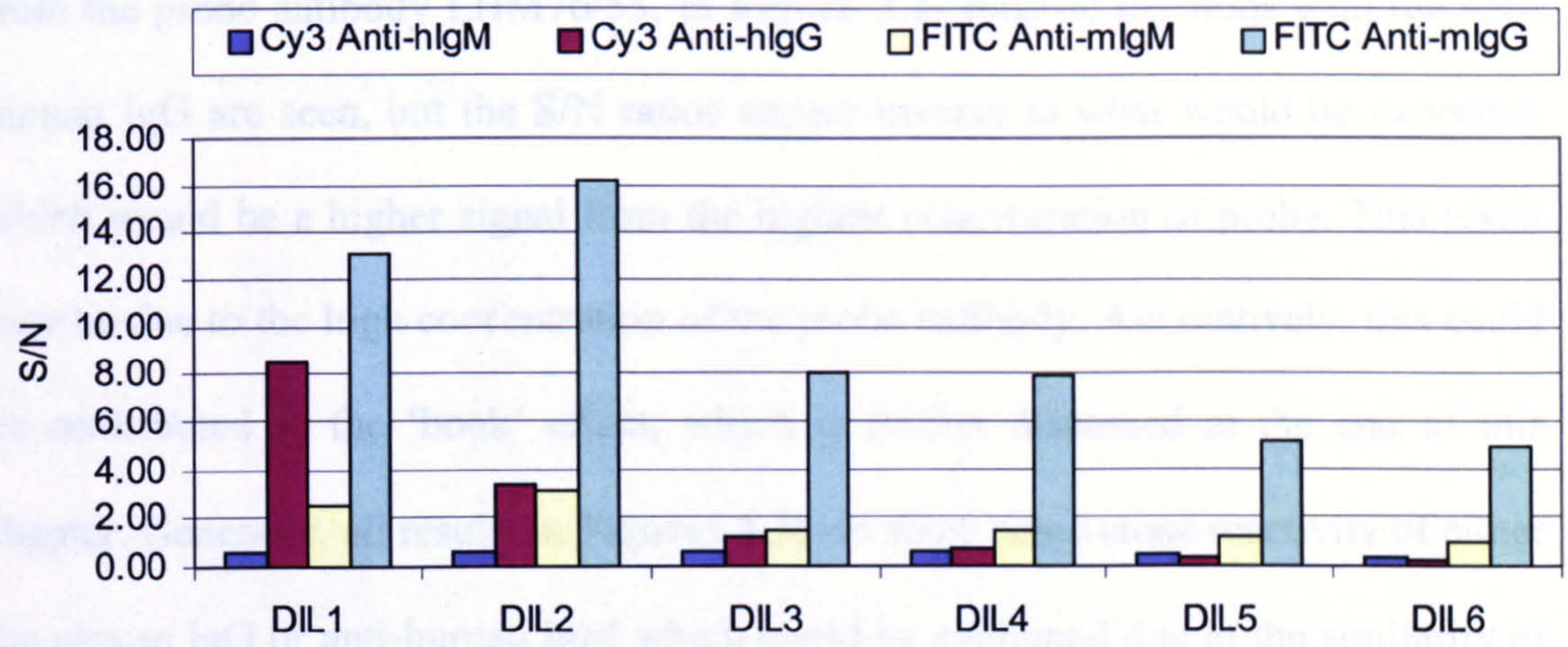



**Figure 3.3f.** Reactions of probe Anti-HIV HIVG1 mouse IgG at various dilutions, with target anti-species antibodies. Slide type poly-L-lysine, slide reps 1, pins 700 $\mu$m, probes Table 3.1, probe reps 2, SPM A; blocker PBS-milk, target/volume: various/25 $\mu$l, incubation 120 min, static, scanning method A.

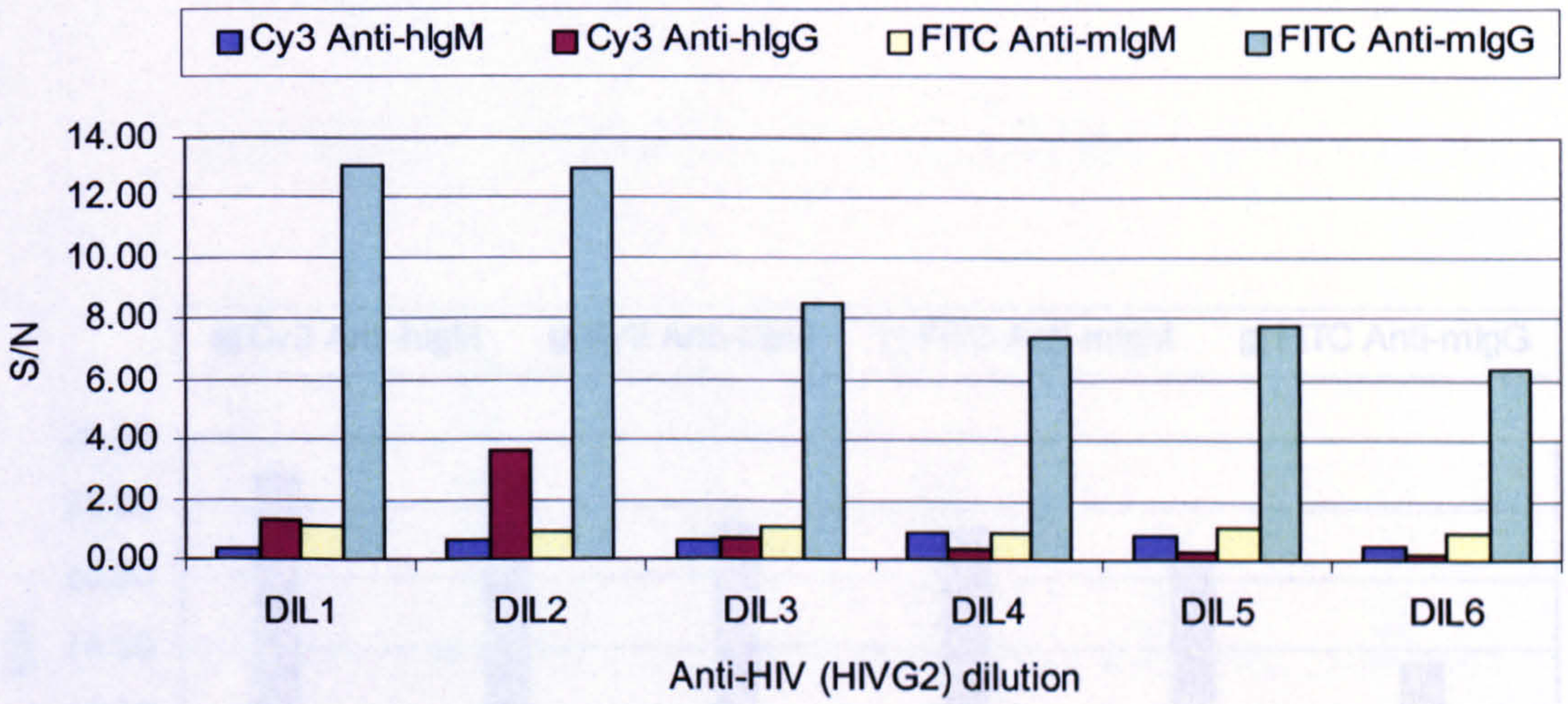



**Figure 3.3g**. Reactions of probe anti-HIV HIVG2 mouse IgG at various dilutions, with target anti-species antibodies. Slide type poly-L-lysine, slide reps 1, pins 700 $\mu$m, probes Table 3.1, probe reps 2, SPM A; blocker PBS-milk, target/volume: various/25 $\mu$l, incubation 120 min, static, scanning method A.

The results from all the human IgG probe antibodies are shown in **Figures 3.3h-m**.

In all cases, specific reactions of probe antibody with the anti-human IgG are shown.

The S/N ration reaches up to 25 in all but one graph, **Figure 3.3l**, showing the results

from the probe antibody LHM76/55. In **Figure 3.3l** specific reactions with the anti-human IgG are seen, but the S/N ratios appear inverse to what would be expected, which would be a higher signal from the highest concentration of probe. This result may be due to the high concentration of the probe antibody. Alternatively, this could be contributed to the 'hook' effect, which is further discussed at the end of this chapter. Generally, all results in **Figures 3.3h-m** show some cross-reactivity of either the mouse IgG or anti-human IgM which could be explained due to the similarity of the antibody structures, and that the antibodies were not anti-species adsorbed during manufacture (supplied by Sigma, U.K.). Also, many of the cell lines (cell line identity prefix LHM, LDM or ESD) are human-mouse hybridomas and may give cross-reactivity with anti-mouse reagents.

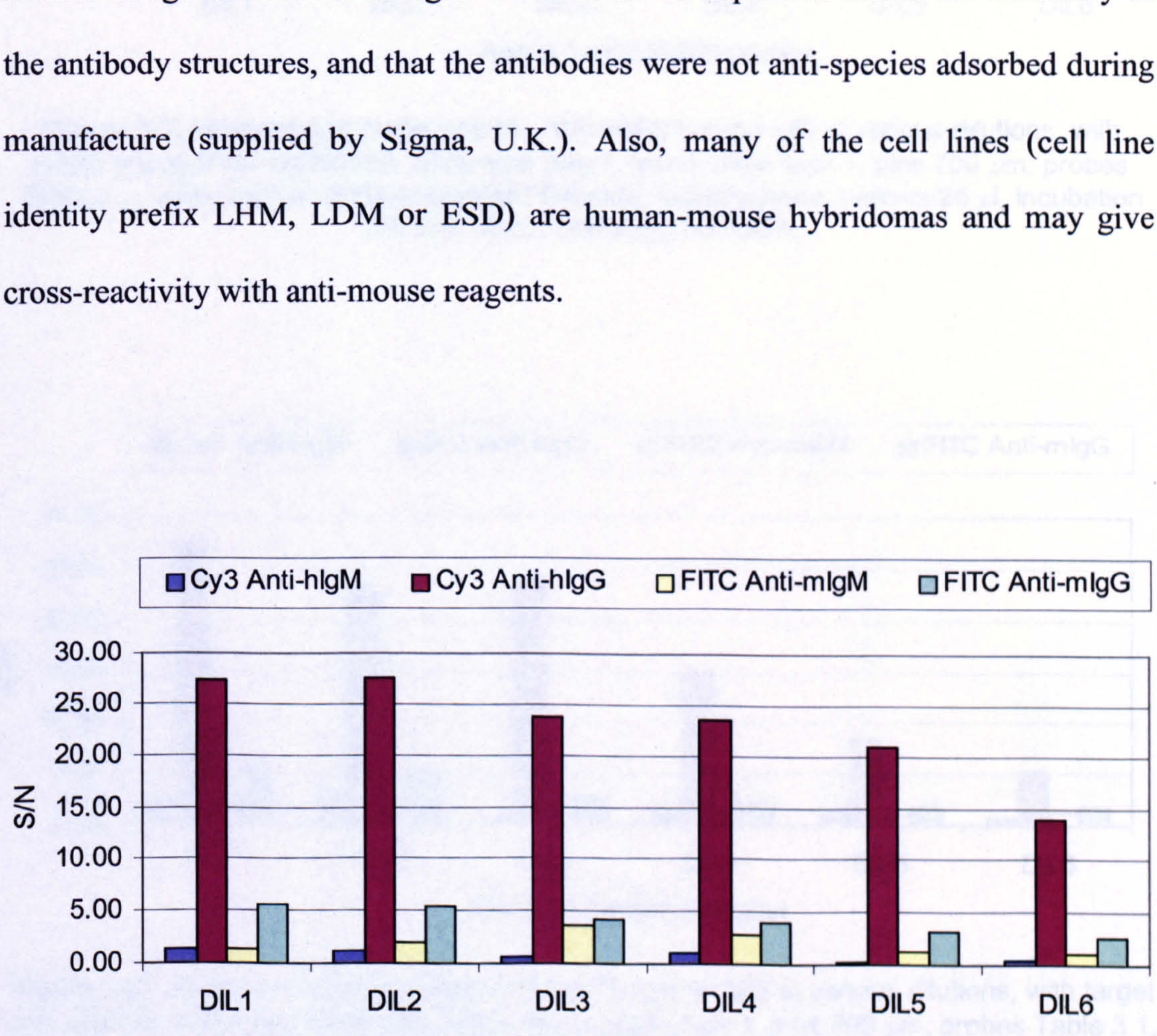



**Figure 3.3h.** Reactions of probe Anti-D ESD1 human IgG at various dilutions, with target anti-species antibodies. Slide type poly-L-lysine, slide reps 1, pins 700 $\mu$m, probes Table 3.1, probe reps 2, SPM A; blocker PBS-milk, target/volume: various/25 $\mu$l, incubation 120 min, static, scanning method A.

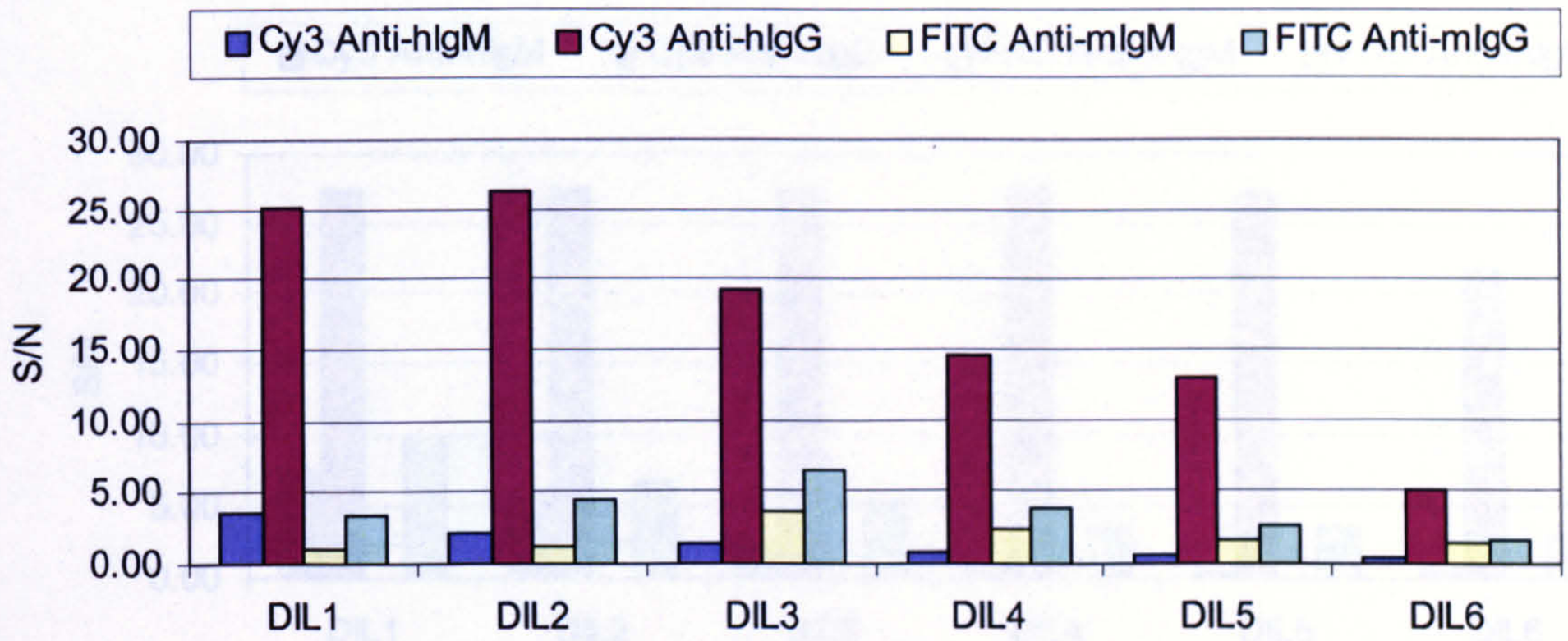



**Figure 3.3i.** Reactions of probe Anti-D LHM169/80 human IgG at various dilutions, with target anti-species antibodies. Slide type poly-L-lysine, slide reps 1, pins 700 $\mu$m, probes Table 3.1, probe reps 2, SPM A; blocker PBS-milk, target/volume: various/25 $\mu$l, incubation 120 min, static, scanning method A.

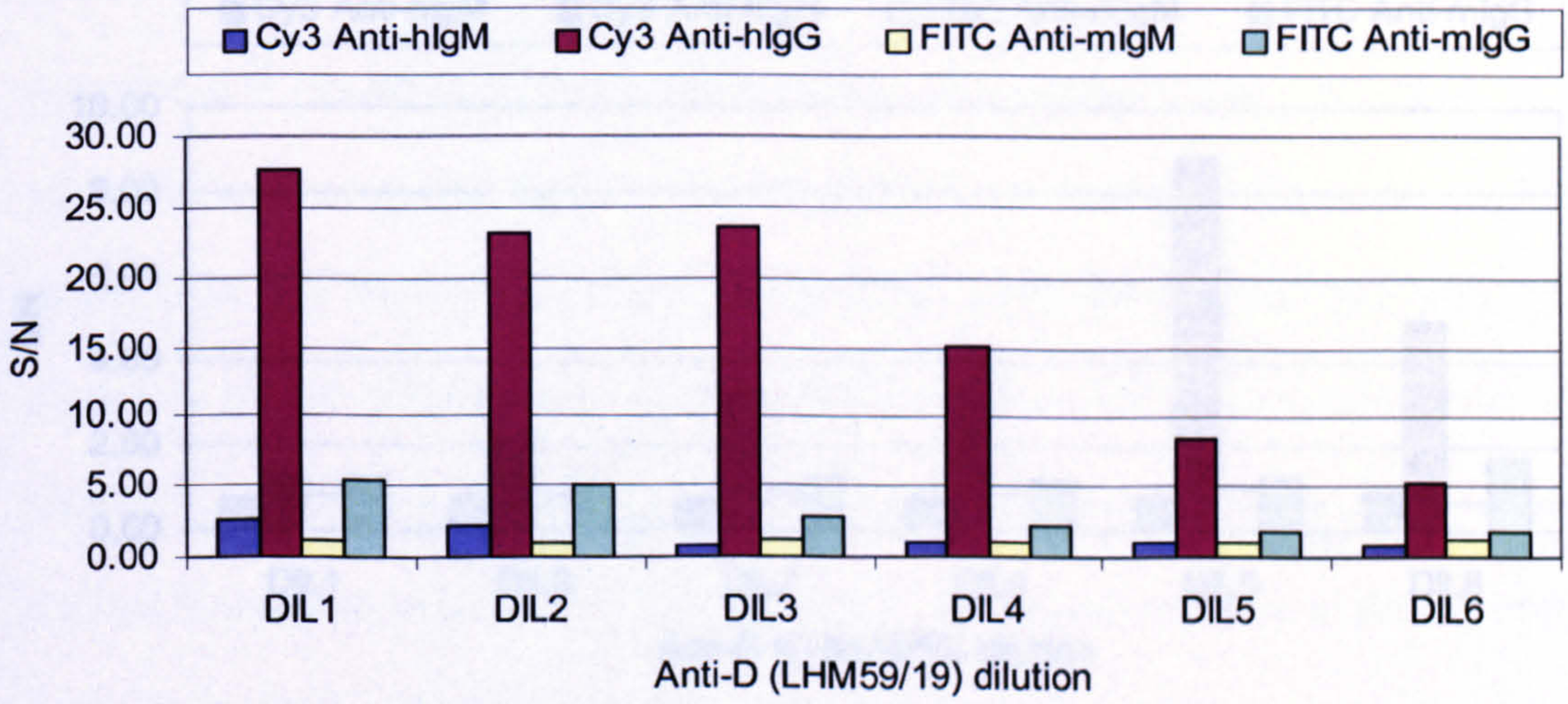



**Figure 3.3j.** Reactions of probe Anti-D LHM59/19 human IgG at various dilutions, with target anti-species antibodies. Slide type poly-L-lysine, slide reps 1, pins 700 $\mu$m, probes Table 3.1, probe reps 2, SPM A; blocker PBS-milk, target/volume: various/25 $\mu$l, incubation 120 min, static, scanning method A.

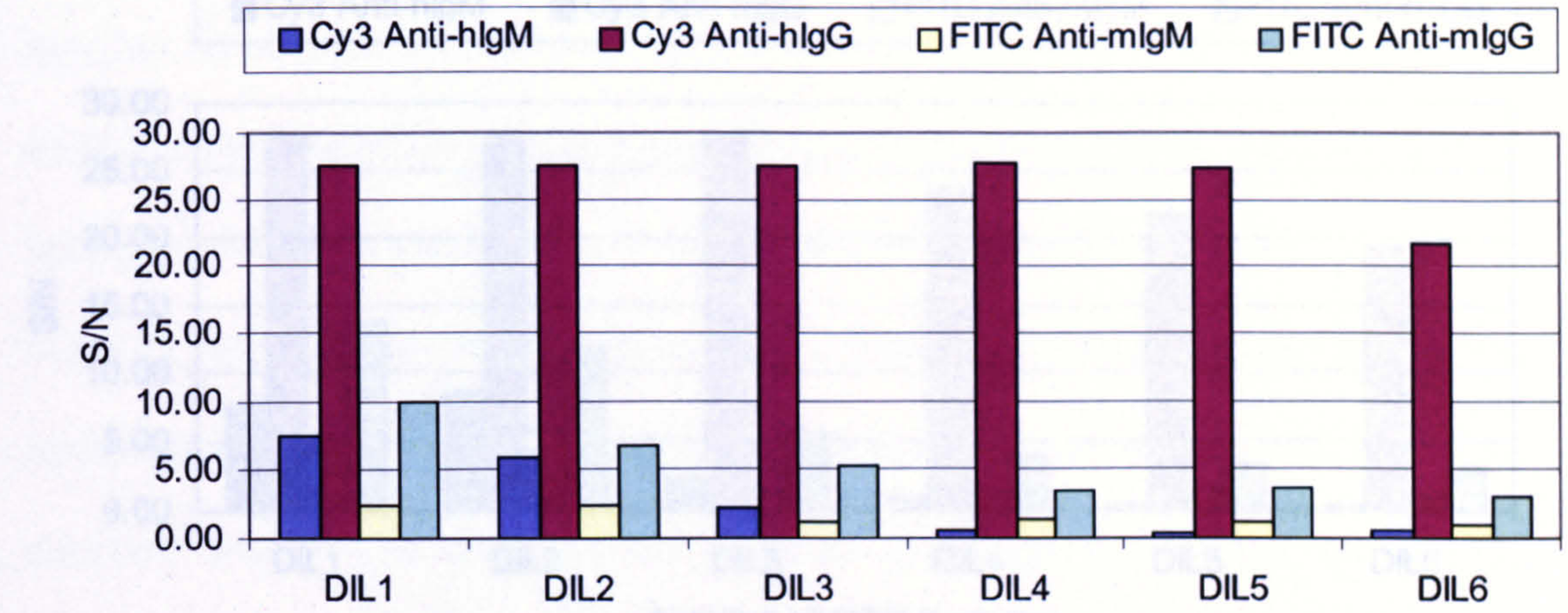



**Figure 3.3k.** Reactions of probe Anti-D LHM70/45 human IgG at various dilutions, with target anti-species antibodies. Slide type poly-L-lysine, slide reps 1, pins 700 $\mu$m, probes Table 3.1, probe reps 2, SPM A; blocker PBS-milk, target/volume: various/25 $\mu$l, incubation 120 min, static, scanning method A.

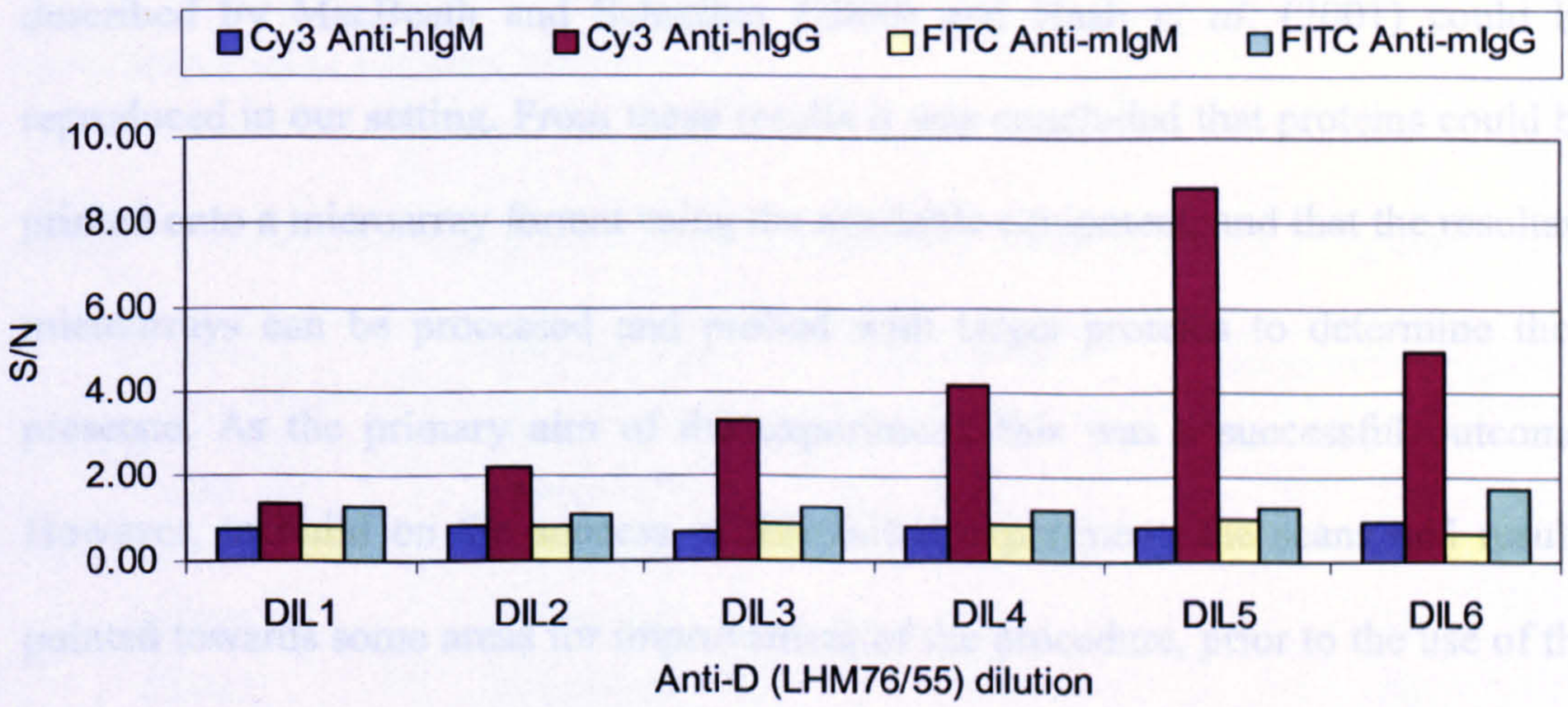



**Figure 3.3l.** Reactions of probe Anti-D LHM76/55 human IgG at various dilutions, with target anti-species antibodies. Slide type poly-L-lysine, slide reps 1, pins 700 $\mu$m, probes Table 3.1, probe reps 2, SPM A; blocker PBS-milk, target/volume: various/25 $\mu$l, incubation 120 min, static, scanning method A.

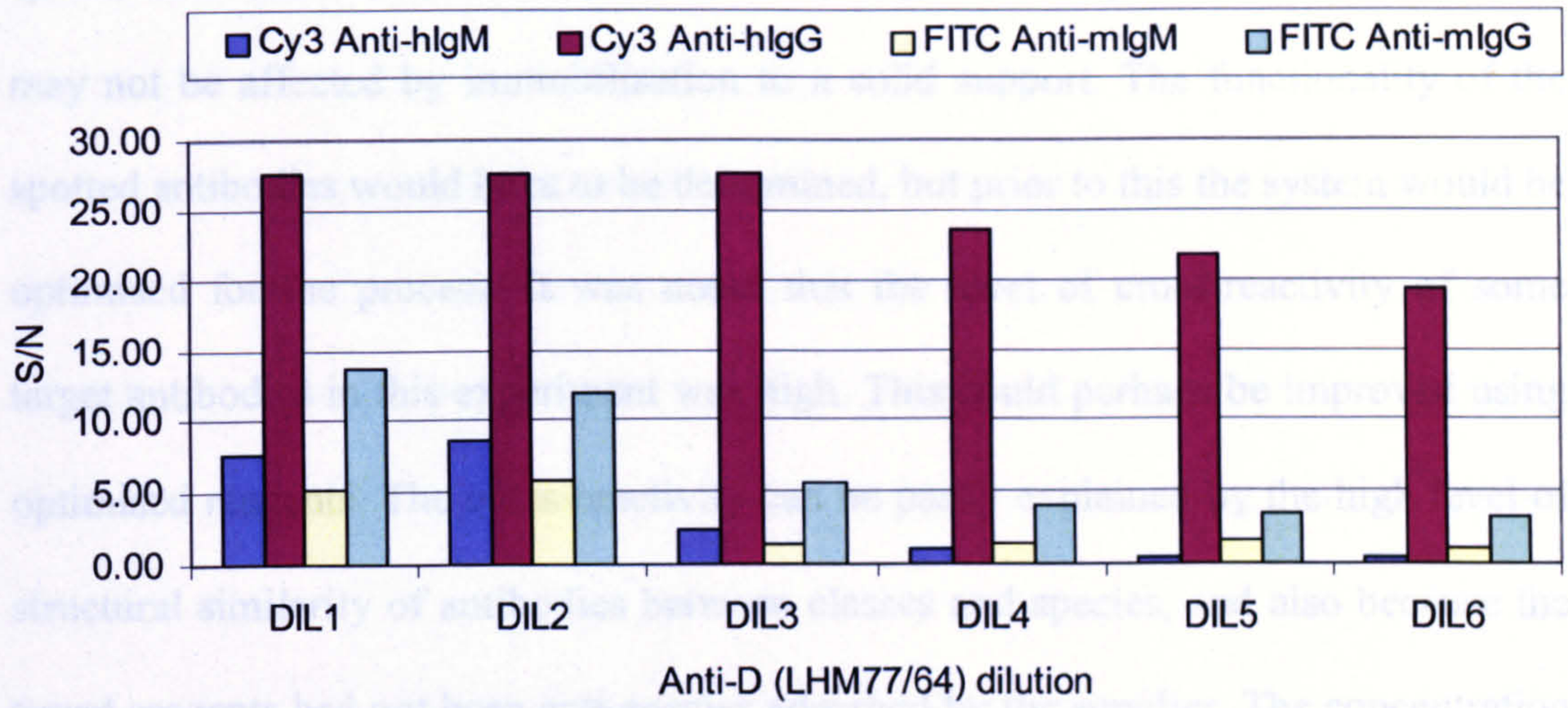



**Figure 3.3m.** Reactions of probe Anti-D LHM77/64 human IgG at various dilutions, with target anti-species antibodies. Slide type poly-L-lysine, slide reps 1, pins 700 $\mu$m, probes Table 3.1, probe reps 2, SPM A; blocker PBS-milk, target/volume: various/25 $\mu$l, incubation 120 min, static, scanning method A.

The aim of this experiment was to determine if the protein microarray procedures described by MacBeath and Schreiber (2000) and Haab *et al.* (2001) could be reproduced in our setting. From these results it was concluded that proteins could be printed onto a microarray format using the available equipment, and that the resultant microarrays can be processed and probed with target proteins to determine their presence. As the primary aim of the experiment, this was a successful outcome. However, to build on the success of this initial experiment, the scans and results pointed towards some areas for improvement of the procedure, prior to the use of the microarrays for the intended purpose of blood typing and antibody screening.

The experiment showed the presence of the probe antibodies on the slides following blocking, sensitisation (interaction phase) and washing procedures. What was not shown by this experiment was whether the probes were still functional on the microarray solid-phase format. The target antigen-binding site of many of the anti-

species antibodies is on the Fc portion of the target antibody molecules, an area that may not be affected by immobilisation to a solid support. The functionality of the spotted antibodies would have to be determined, but prior to this the system would be optimised for the process. It was noted that the level of cross-reactivity of some target antibodies in this experiment was high. This could perhaps be improved using optimised reagents. The cross-reactivity can be partly explained by the high level of structural similarity of antibodies between classes and species, and also because the target reagents had not been anti-species adsorbed by the supplier. The concentration of target antibodies was not optimised prior to this experiment and it may be that adjusted levels would give more specific results. The specificity of the target antibodies was demonstrated clearly when graphs were plotted of each target against all the probes at similar concentrations (see **Table 3.3**), using the same data from that used above, as in **Figures 3.4a-d.**

**Table 3.3.** Dilution of probe proteins used for analysis of target antibodies in **Figures 3.4a-d.**

| Protein ID | Protein/Ab type | Dilution Used | Concentration (µg/ml) |
|---|---|---|---|
| ES15 | mouse IgM | DIL3 | 42 |
| ES9 | mouse IgM | DIL2 | 54 |
| ESD1 | human IgG | DIL5 | 50 |
| HIVG1 | mouse IgG | DIL5 | 50 |
| HIVG2 | mouse IgG | DIL5 | 50 |
| LA2 | mouse IgM | DIL1 | 55 |
| LB2 | mouse IgM | DIL2 | 72 |
| LDM3 | human IgM | DIL1 | 6.4 |
| LHM169/80 | human IgG | DIL5 | 50 |
| LHM59/19 | human IgG | DIL5 | 50 |
| LHM70/45 | human IgG | DIL5 | 50 |
| LHM76/55 | human IgG | DIL5 | 50 |
| LHM77/64 | human IgG | DIL5 | 50 |

The results show that concentration of the target reagents anti-mouse IgG and anti-mouse IgM would require adjustment to give enhanced reactions, but it is unlikely that a change in concentration would increase or decrease cross reactivity. This is analogous to results found in Haab *et al.* (2001). They reported that a variation in fluorescent target labelling/activity is a prominent source of variability in fluorescence measurements, and this will be further discussed at the end of this chapter.

**Figures 3.4a-d** show the results from the separate slides from individual target antibodies. Although a very low level of LDM3 was spotted onto the microarray represented in **Figure 3.4a**, the target anti-human IgM still detects it and gives the highest level of S/N. Cross reactivity with a mouse IgM probe (ES9) is evident.

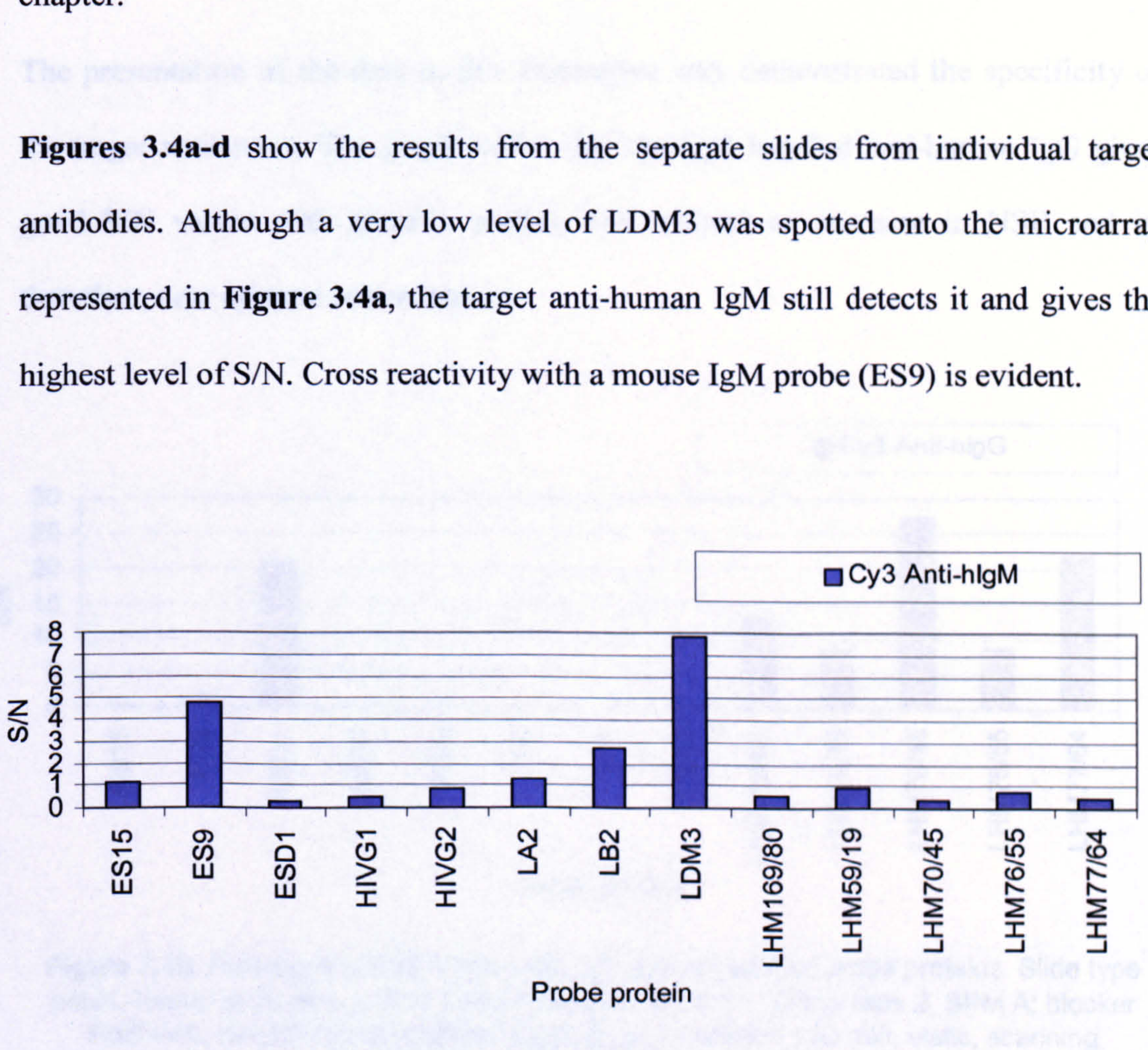



**Figure 3.4a.** Reactions of Cy3 anti-human IgM against spotted probe proteins. Slide type poly-L-lysine, slide reps 1, pins 700 $\mu$m, probes Table 3.1, probe reps 2, SPM A; blocker PBS-milk, target/volume: Cy3 anti-hIgM/25 $\mu$l, incubation 120 min, static, scanning method A.

In **Figure 3.4b** it is clear that the target anti-human IgG gives specific reactions from known human IgG probe antibodies. In **Figure 3.4c** the highest S/N values are seen with ES15, ES9, LA2 and LB2, which are all mouse IgM antibodies. **Figure 3.4d** shows the reactions of the target anti-mouse IgG. Here, a very similar pattern to that found in **Figure 3.4c** is seen. The reactions with the mouse IgG antibody probes are lower than those of mouse IgM antibody probes.

The presentation of the data in this alternative way demonstrated the specificity of the target antibodies. The graph shows that the Cy3 labelled anti-human IgG gives good S/N values with specific probes, and without an increase in NSB and is, therefore, near optimal concentration.

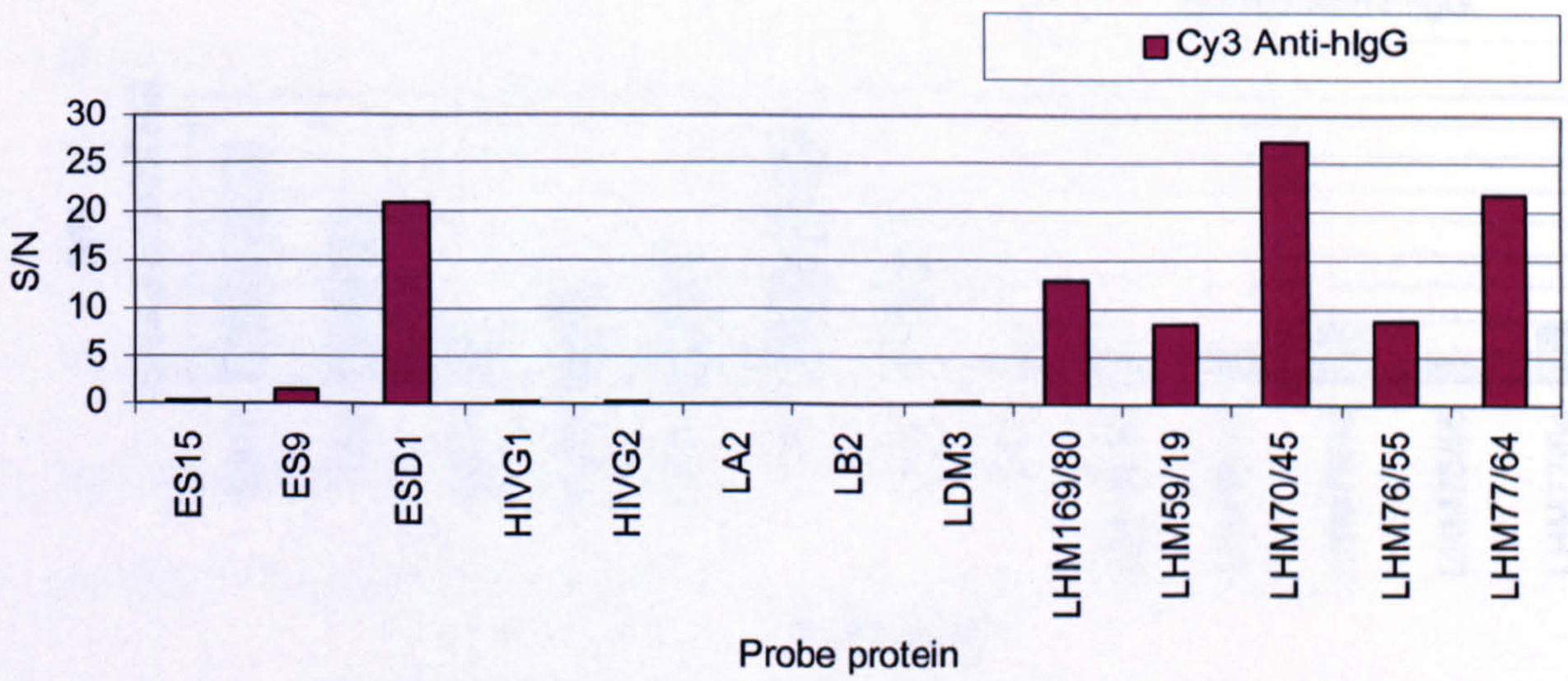



**Figure 3.4b.** Reactions of Cy3 anti-human IgG against spotted probe proteins. Slide type poly-L-lysine, slide reps 1, pins 700 $\mu$m, probes Table 3.1, probe reps 2, SPM A; blocker PBS-milk, target/volume: Cy3 anti-hIgG/25 $\mu$l, incubation 120 min, static, scanning method A.

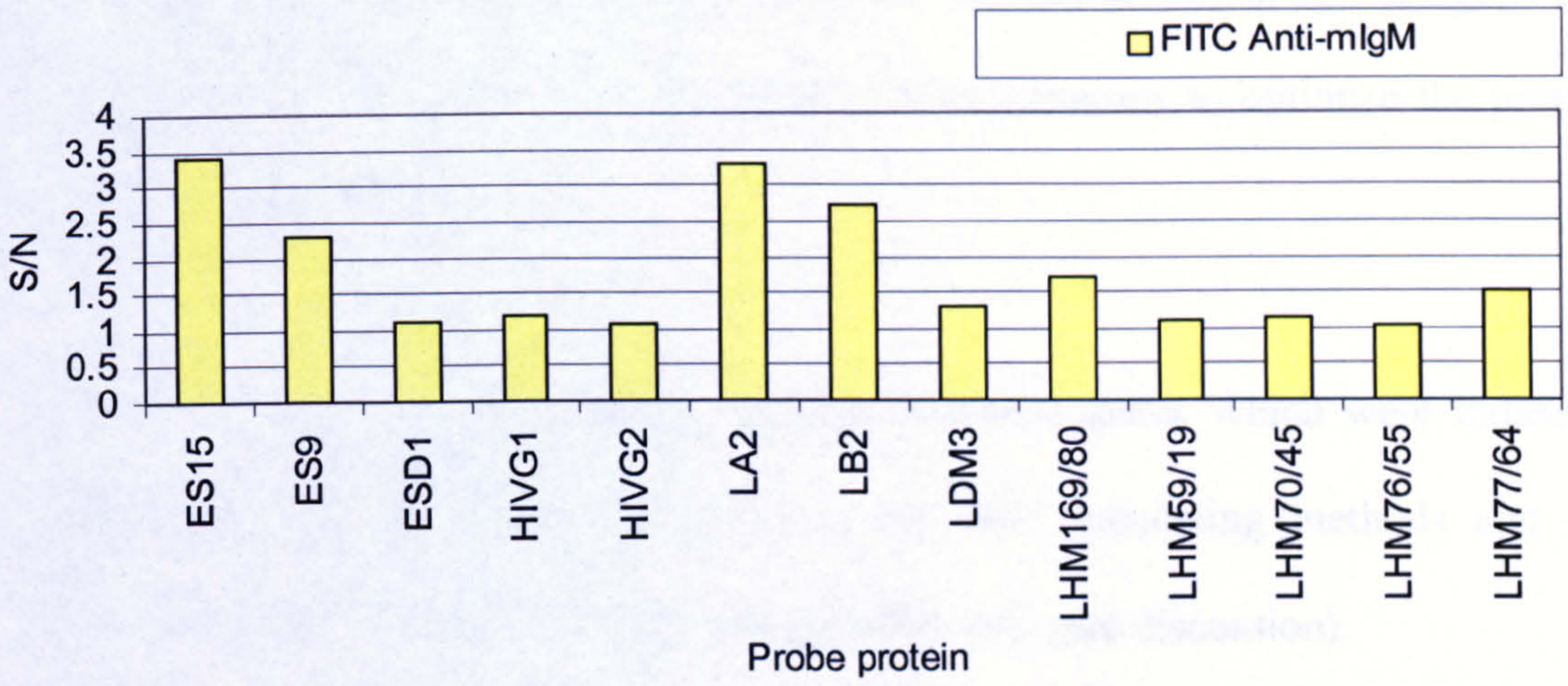



**Figure 3.4c.** Reactions of FITC anti-mouse IgM against spotted probe proteins. Slide type poly-L-lysine, slide reps 1, pins 700 $\mu$m, probes Table 3.1, probe reps 2, SPM A; blocker PBS-milk, target/volume: FITC anti-mIgM/25 $\mu$l, incubation 120 min, static, scanning method A.

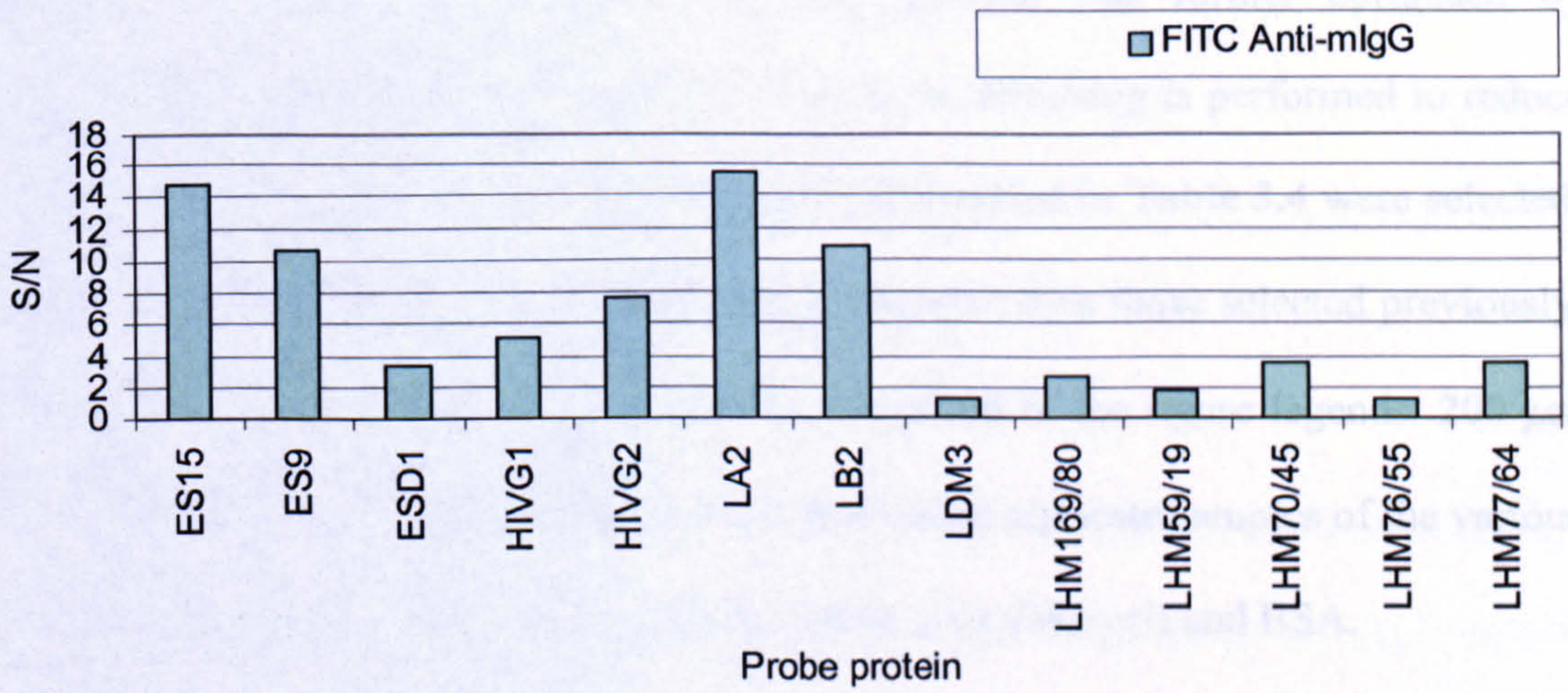



**Figure 3.4d**. Reactions of FITC anti-mouse IgG against spotted probe proteins. Slide type poly-L-lysine, slide reps 1, pins 700 $\mu$m, probes Table 3.1, probe reps 2, SPM A; blocker PBS-milk, target/volume: FITC anti-mIgG/25 $\mu$l, incubation 120 min, static, scanning method A.

Given the specificity of the reactions between the sets of human IgG antigens and antibodies, these were selected for subsequent experiments to optimise the protein microarray procedure.

This experiment had demonstrated some problematic areas, which were indicated previously. Improvements were made to the slide processing methods and the analysis method developed to give meaningful results (see discussion).

## 3.3 Optimisation of Protein Microarray Performance: Blocking Methods and Detergents

### 3.3.1 Optimisation of the Microarray Blocking Procedure

In this experiment, the protein microarray process was further optimised by investigating the blocking of the microarray slides. Blocking is performed to reduce NSB. For this purpose, the set of probe proteins detailed in **Table 3.4** were selected. This probe set allows for far more varied interactions than those selected previously. The probes were printed and processed as described in the figure legends. 200 $\mu$m pins were used in this experiment to allow many (21) replicate samples of the various probes. Two blockers were investigated, non-fat milk (Marvel) and BSA.

Cy3 labelled anti-human IgG was selected as a target/detection reagent, as it was shown to have worked well in the previous experiment. Cy3 labelled rProtein-L was also used here for detection of probes. rProtein-L (recombinant) has been shown to bind strongly to kappa light chains on human antibodies as well as those from rat and mouse (actigen.com), but should not bind to bovine, sheep or goat antibodies. The

rProtein-L was labelled with Cy3 using the method for Fluorescent Labelling of Antibody/Antigen/Lectin. The target solutions were prepared in the blocking solution as used for each slide.

**Table 3.4** Probe proteins spotted in blocking protein microarray experiment, concentration in $\mu$g/ml.

| Protein ID | Protein/Ab type | Conc. DIL1 | Conc. DIL2 | Conc. DIL3 | Conc. DIL4 | Conc. DIL5 |
|---|---|---|---|---|---|---|
| mIgG | mouse IgG | 500 | 250 | 125 | 62.5 | 31.25 |
| mIgM | mouse IgM | 500 | 250 | 125 | 62.5 | 31.25 |
| rIgG | rabbit IgG | 500 | 250 | 125 | 62.5 | 31.25 |
| sIgG | sheep IgG | 500 | 250 | 125 | 62.5 | 31.25 |
| gIgG | goat IgG | 500 | 250 | 125 | 62.5 | 31.25 |
| HS | human serum | 3000 | 1500 | 750 | 375 | 187.5 |
| BS | bovine serum | 3000 | 1500 | 750 | 375 | 187.5 |

As this experiment aimed to evaluate the effect of blocking to improve specific reactions and reduce non-specific reactions, the values used to calculate noise were those from PBS spots.

Microarrays processed using BSA blocking showed a high level of comet tailing. The scan images in **Figure 3.5** show the difference in the level of comet tailing found in scans from BSA blocking compared to non-fat milk blocking, where no comet tailing was seen. The graphical results are presented in **Figures 3.6a-b**. Error bars demonstrate the variability between replicate spots on the microarrays.

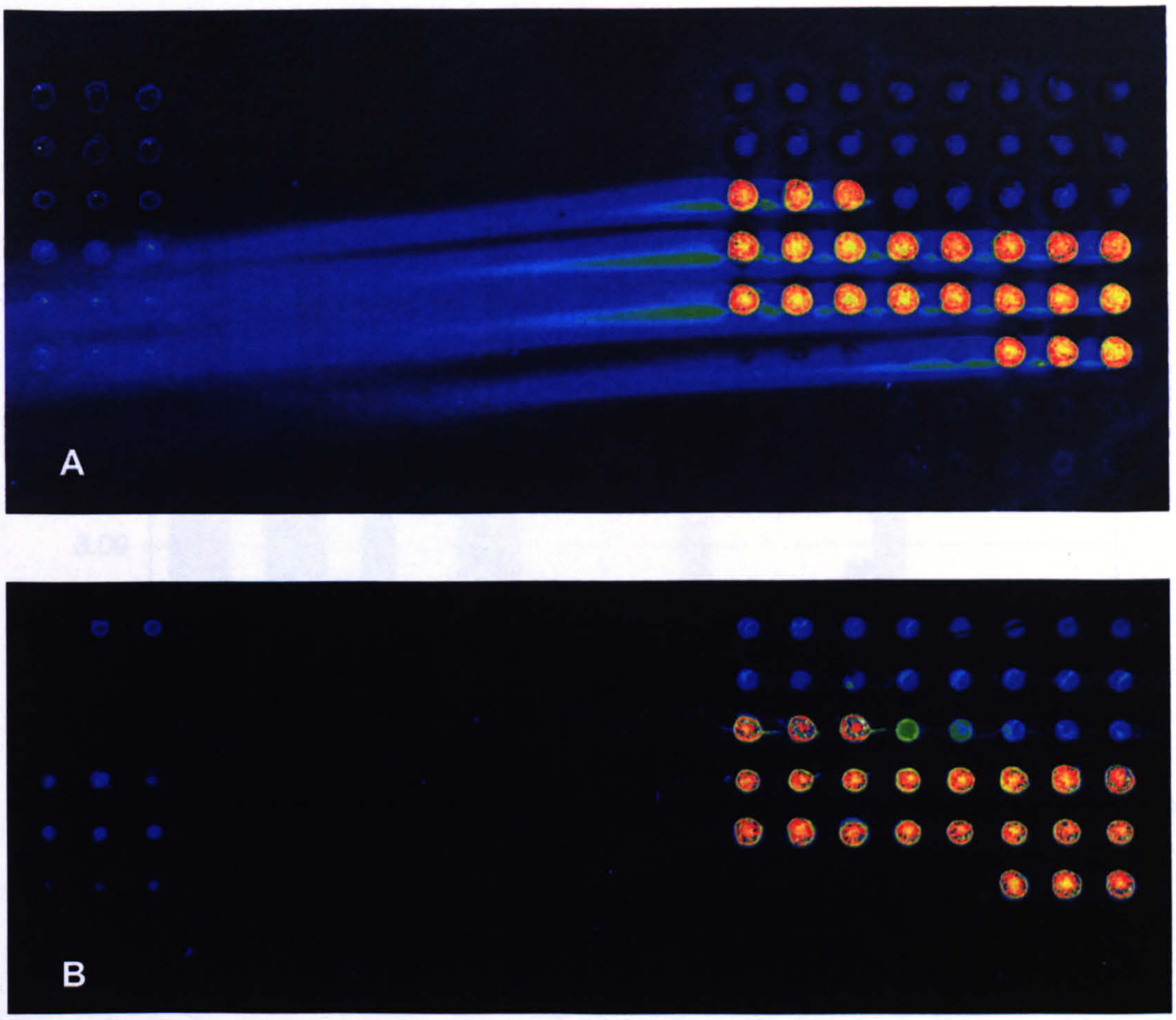



**Figure 3.5**. Pseudo colour scan images to determine the blocking procedure for protein microarrays. Slide type poly-L-lysine, slide reps 1, pins 200 $\mu$m, probes Table 3.4, probe reps 21, Fig A,SPM A; blocker PBS-milk, Fig B, SPM B; blocker PBS-BSA, target/volume: Cy3 anti-hIgG/25 $\mu$l, incubation 120 min, static, scanning method A.

**Figure 3.6a** demonstrates a higher binding (S/N) when using milk blocking. Although it also demonstrates higher cross reactivity with rabbit IgG, this method was selected for future processing. Only the data from human serum and rabbit IgG is presented as all other data gave very low S/N. The lack of results from HS DIL3 was investigated and found to have resulted from insufficient reagent to deposit spots on this particular slide.

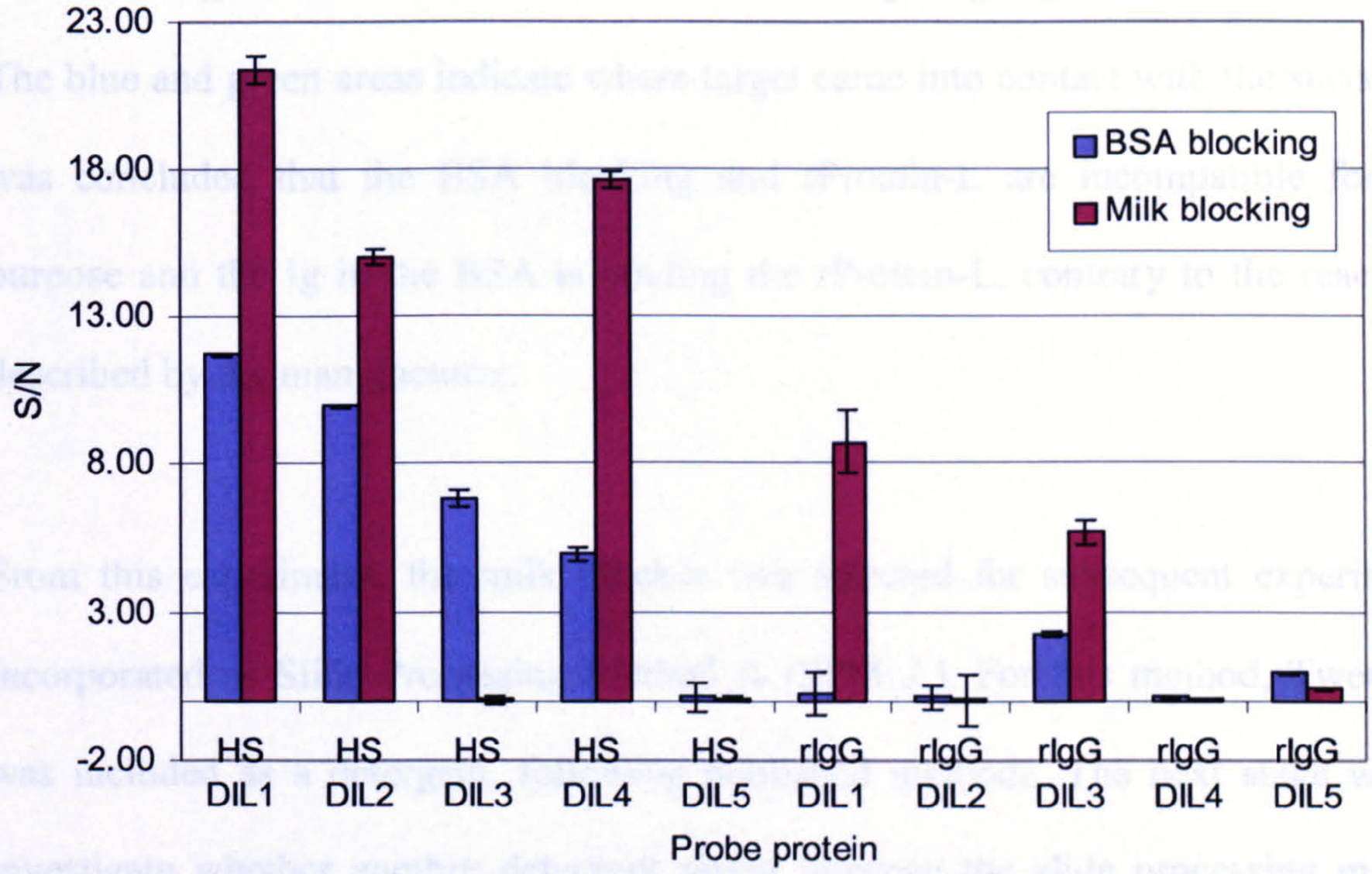



**Figure 3.6a**. Comparison of BSA and Milk blocking using reactions of Cy3 anti-human IgG against spotted probe proteins. Slide type poly-L-lysine, slide reps 1, pins 200 $\mu$m, probes Table 3.4, probe reps 21, SPM A; blocker PBS-milk, SPM B; blocker PBS-BSA, target/volume: Cy3 anti-hIgG/25 $\mu$l, incubation 120 min, static, scanning method A.

**Figure 3.6b** shows the results obtained from the milk-blocked slide to which Cy3 rProtein-L was added as target. The results seen in the figure show low levels of binding, but also that higher S/N ratios are evident against human serum, which would be expected to bind the rProtein-L. This reagent had potential as a quality control reagent to determine antibody levels retained on the microarrays at various stages of processing but S/N values are low. This reagent may be giving low S/N values because of insufficient labelling as the labelling ratio was calculated as 1.45 dye molecules per rProtein-L molecule.

The reactions from the BSA blocked slide with target Cy3 rProtein-L gave such low quality data (when background values compared to signal intensity values as described in Chapter 2) that the data was not analysed. A colour image of the scan is

shown in **Figure 3.6c.** The entire reaction area is giving high fluorescence signals. The blue and green areas indicate where target came into contact with the surface. It was concluded that the BSA blocking and rProtein-L are incompatible for this purpose and the Ig in the BSA is binding the rProtein-L, contrary to the reactivity described by the manufacturer.

From this experiment, the milk blocker was selected for subsequent experiments incorporated as Slide Processing Method A (SPM A). For this method, Tween 20 was included as a detergent, following published methods. The next stage was to investigate whether another detergent might improve the slide processing method further.

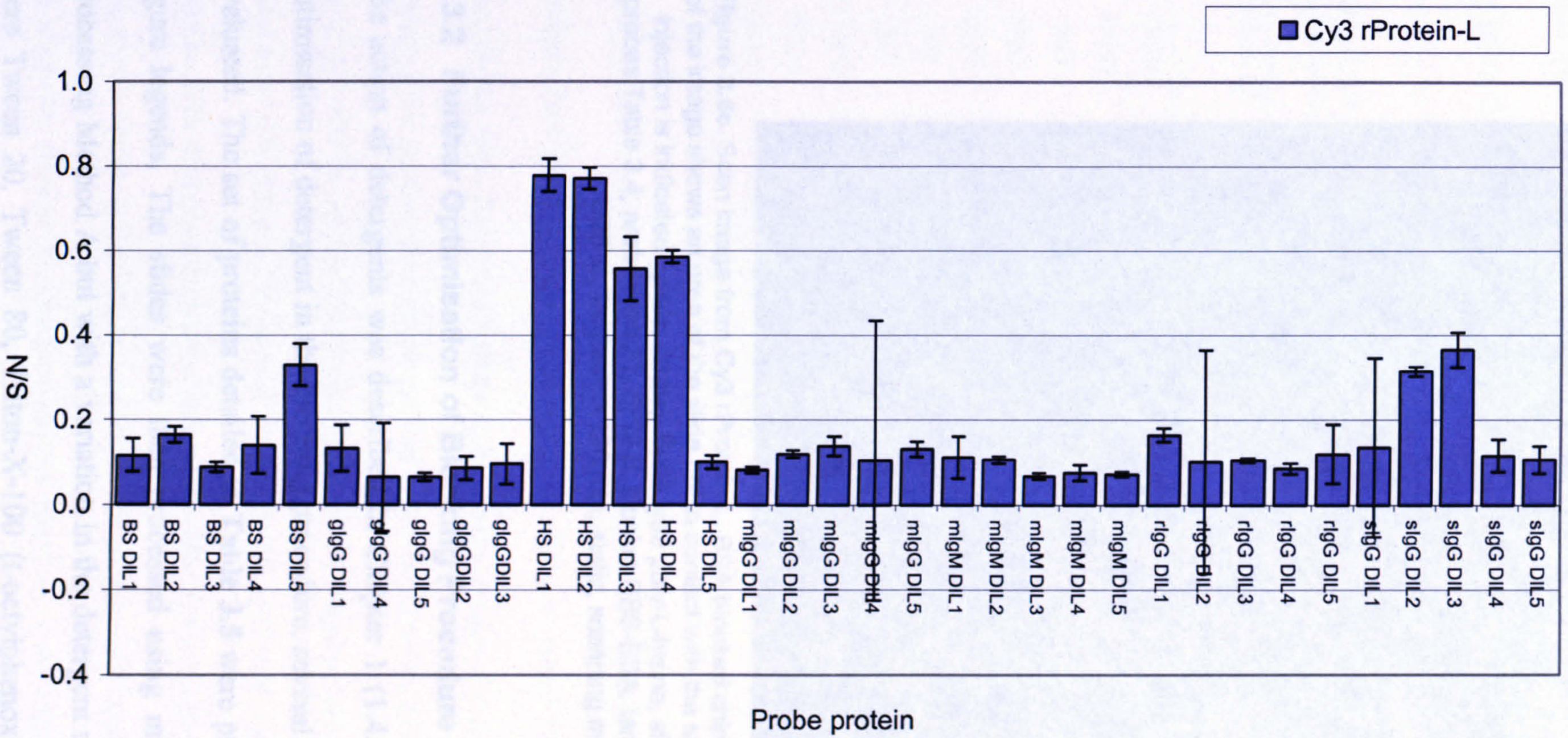



**Figure3.6b.** Reactions of Cy3 rProtein-L against spotted probe proteins, using milk blocking.
Slide type poly-L-lysine, slide reps 1, pins 200 $\mu$m, probes Table 3.4, probe reps 21, SPM A; blocker PBS-milk, target/volume: Cy3 rProtein-L/25 $\mu$l, incubation 120 min, static, scanning method A.

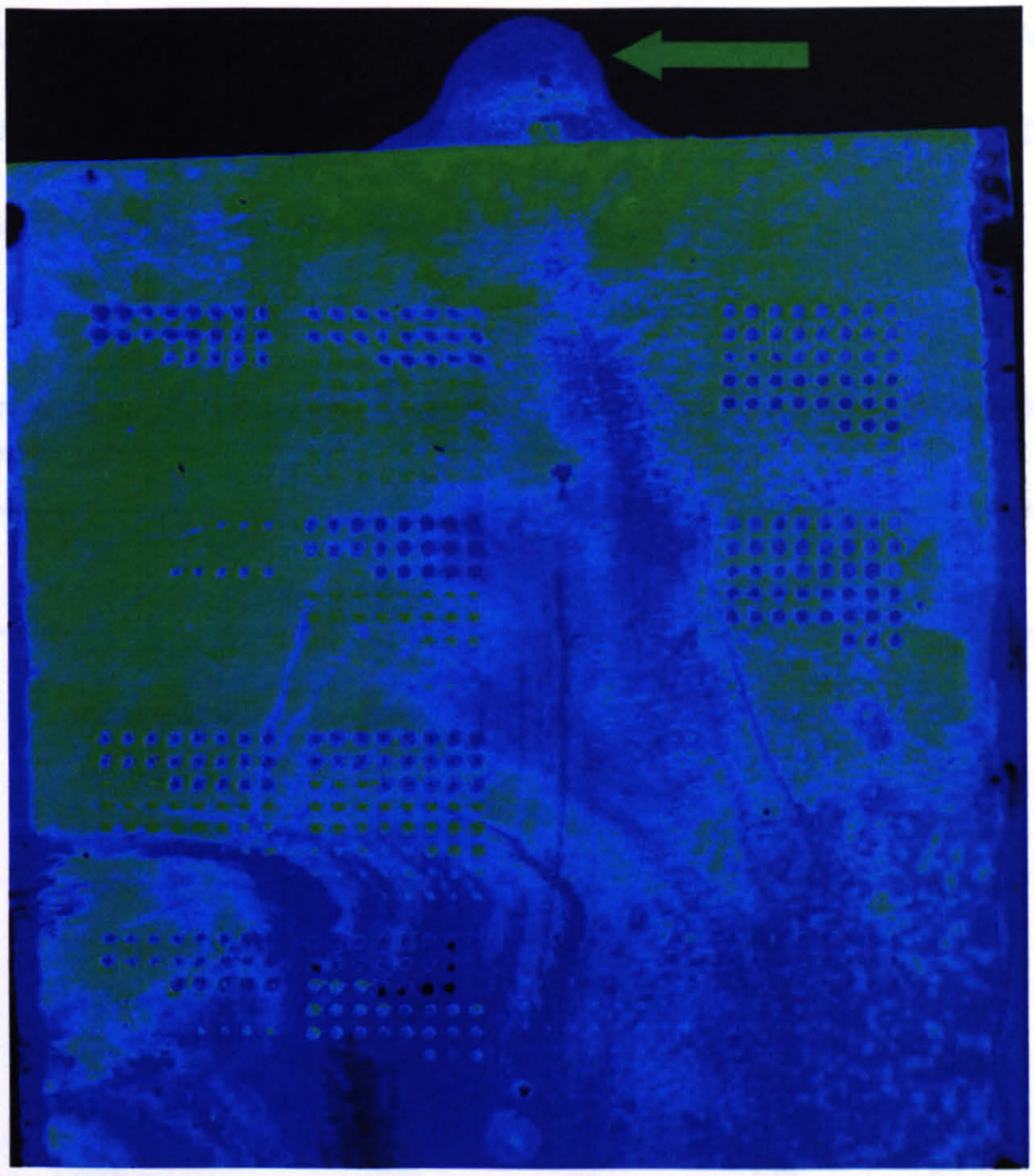


**Figure 3.6c.** Scan image from Cy3 rProtein-L, BSA blocked chip. The black area at the top of the image shows an area of the slide not in contact with the target solution. The point of injection is indicated (green arrow). Slide type poly-L-lysine, slide reps 1, pins 200 $\mu$m, probes Table 3.4, probe reps 21, SPM B; blocker PBS-BSA, target/volume: Cy3 rProtein-L/25 $\mu$l, incubation 120 min, static, scanning method A.

### 3.3.2 Further Optimisation of Blocking Procedure Using Detergents

The action of detergents was described in Chapter 1 (1.4.2.4). For the purpose of optimisation of detergent in the blocking procedure, several different detergents were evaluated. The set of proteins detailed in **Table 3.5** were printed as described in the figure legends. The slides were then processed using milk blocking as in Slide Processing Method A but with a variation in the detergent used. The detergents used were Tween 20, Tween 80, Triton-X-100 (t-octylphenoxypolyethoxyethanol) and CHAPS (3-[3-cholamidopropyl dimethylammonio]-1-propanesulfonate). Each was

used at concentrations of 0.1 and 0.5 % in solution. These replaced Tween 20 in Slide Processing Method A. The target solution was Cy3 anti-human IgG at 33.33 $\mu$g/ml in the blocking solution without detergent.

**Table 3.5** Proteins spotted in protein microarray experiment to optimise detergent, concentration in $\mu$g/ml.

| Protein ID | Protein/Ab type | Conc. DIL1 | Conc. DIL2 | Conc. DIL3 | Conc. DIL4 | Conc. DIL5 |
|---|---|---|---|---|---|---|
| mIgG | mouse IgG | 500 | 250 | 125 | 62.5 | - |
| mIgM | mouse IgM | 500 | 250 | 125 | 62.5 | - |
| rIgG | rabbit IgG | 500 | 250 | 125 | 62.5 | - |
| sIgG | sheep IgG | 500 | 250 | 125 | 62.5 | - |
| gIgG | goat IgG | 500 | 250 | 125 | 62.5 | - |
| HS | human serum | 3000 | 1500 | 750 | 375 | 187.5 |
| BS | bovine serum | 3000 | 1500 | 750 | 375 | 187.5 |
| LDM3 | human IgM | 6.4 | 3.2 | 1.6 | 0.8 | - |
| ESD1 | human IgG | 500 | 250 | 125 | 62.5 | - |
| HIVG2 | mouse IgG | 500 | 250 | 125 | 62.5 | - |

During development of this protein microarray process, the use of appropriate controls had become more apparent. For the previous experiment PBS spots were used to calculate the noise values as PBS represented the spotting buffer in which all probes were prepared. Further probes were included in this experiment to allow for more suitable biological controls. As shown in **Figure 3.4b,** the Cy3 anti-human IgG gave low S/N ratio with human IgM, mouse IgG and mouse IgM probes, but in **Figure 3.6a** is shown to cross-react with rabbit IgG. It has also been shown to cross react with sheep IgG (data not shown). An appropriate biological negative control is an antibody similar in structure and/or species to the expected positive probe, but

which is directed to an unrelated antigen that would not be present in the target solution.

The large amount of data generated from this experiment was, therefore, analysed in two ways. All spots except those from human serum, human IgG (ESD1), rabbit IgG and sheep IgG were used to calculate the noise for the S/N ratios demonstrated in **Figure 3.7a**. For the data in **Figure 3.7b,** all data apart from human serum and human IgG (ESD1) were used to calculate S/N.

The anti-human IgG reacted specifically with the monoclonal anti-D ESD1 and the human serum (**Figure 3.7a**). Although while showing cross-reactivity with rabbit IgG, cross-reactivity with sheep IgG is not present. In **Figure 3.7b,** the data looks quite different and far lower S/N values are seen (error bars are not appended). This is because the expected negative probes have been used to calculate noise and this includes rabbit IgG. Therefore, the noise value is far higher and means resultant S/N values are far lower.

Theoretically, a higher S/N ratio in this experiment could mean that noise had been reduced therefore resulting in a higher ratio, or that more specific binding had taken place. From this experiment it was decided that no alternative detergent gave improved results that justified a change from the use of Tween 20.

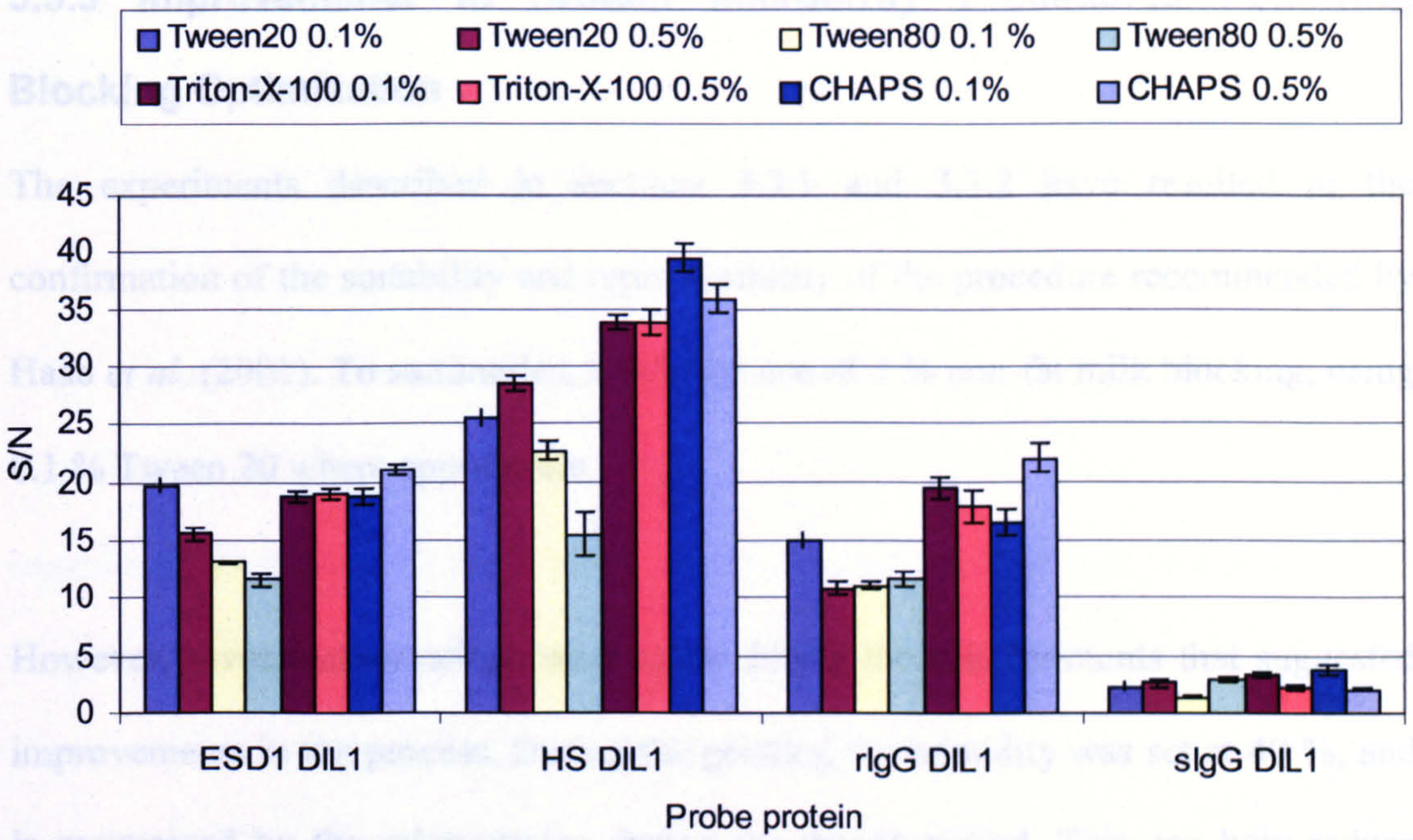



**Figure 3.7a.** Comparison of different detergents in the blocking solution using reactions of Cy3 anti-human IgG against spotted probe proteins, with all other probes used to calculate S/N values. Results are from Dilution 1 only. Slide type poly-L-lysine, slide reps 1, pins 200 $\mu$m, probes Table 3.5, probe reps 8, SPM A; blocker PBS-milk, target/volume: Cy3 anti-hIgG/25 $\mu$l, incubation 120 min, static, scanning method A.

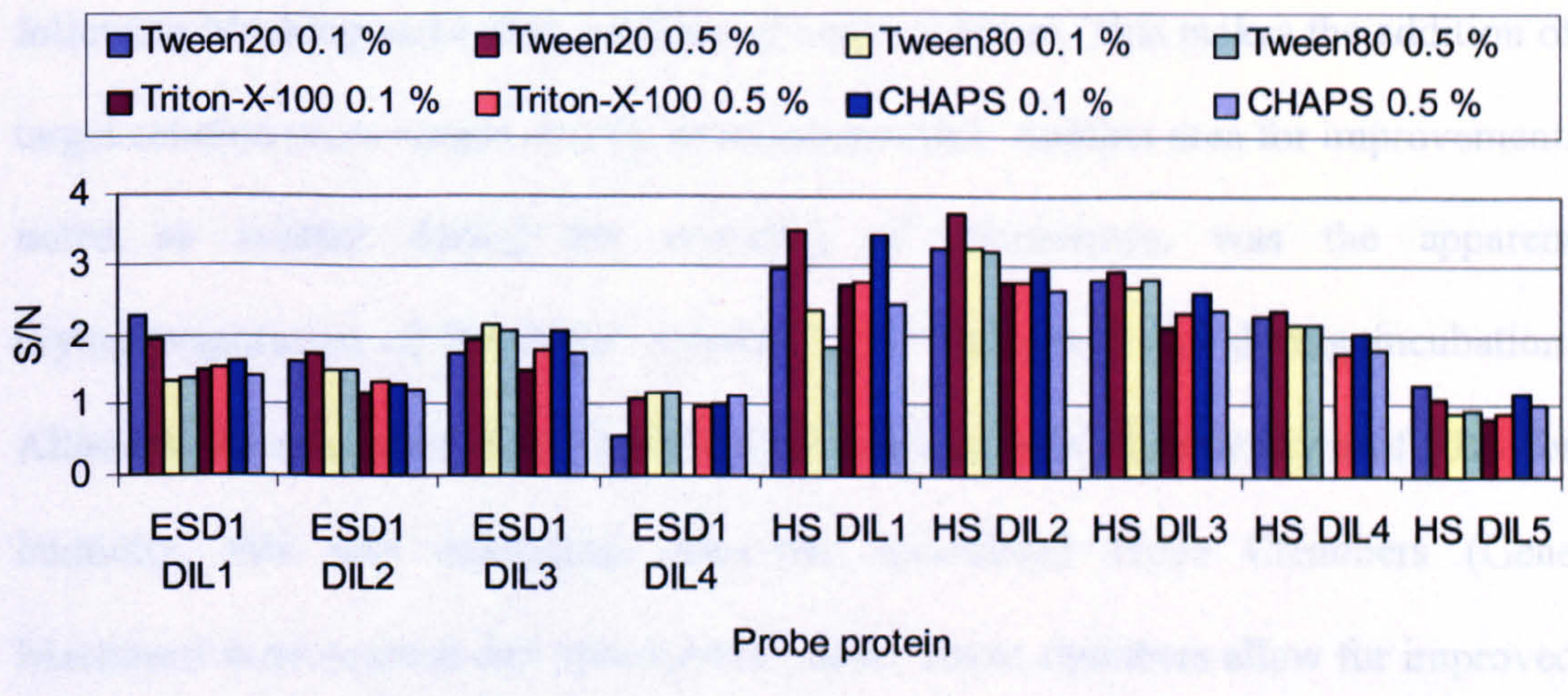



**Figure 3.7b.** Comparison of different detergents in the blocking solution using reactions of Cy3 anti-human IgG against spotted probe proteins. The figure shows the median of each sample S/N calculated from all other protein probes, against each detergent. Slide type poly-L-lysine, slide reps 1, pins 200 $\mu$m, probes Table 3.5, probe reps 8, SPM A; blocker PBS-milk, target/volume: Cy3 anti-hIgG/25 $\mu$l, incubation 120 min, static, scanning method A.

### 3.3.3 Improvements to Protein Microarray Procedures Following Blocking Optimisation

The experiments described in sections 3.3.1 and 3.3.2 have resulted in the confirmation of the suitability and reproducibility of the procedure recommended by Haab *et al.* (2001). To summarise, this is the use of 3 % non-fat milk blocking, using 0.1 % Tween 20 where appropriate.

However, several observations were made during these experiments that suggested improvements in the process. During the printing the humidity was set at 40 %, and is maintained by the microarrayer during the drying period. This can help reduce doughnut shaped spots. To avoid lifting off of the spots during processing, it was felt that a re-hydration step should be included prior to slide blocking. As mentioned previously, customised containers were included for the processing steps, which made processing easier. In addition to this the slides were centrifuged to dryness following blocking and before addition of target solution. This makes the addition of target solution more simple and the processing easier. Another area for improvement, noted as evident during the scanning of microarrays, was the apparent drying/evaporation of the target solution under the cover slip during incubation. Although the microarrays had been sealed in a box with an underlayer of PBS for humidity, this was inadequate therefore specialised Hybe Chambers (Gene Machines) were sourced and subsequently used. These chambers allow for improved sealing of the environment and therefore eliminate evaporation. It was also noted that the immersion of microarrays during washing must be quite rigorous to minimise residual unwanted solution on the slide surface.

All of the improvements summarised above were applied to the original method (Slide Processing Method A) and this is described in Chapter 2 as Slide Processing Method C (SPM C). This new process was used in subsequent experiments.

## 3.4 Confirmation of Optimised Protein Microarray Procedure

Repeatability is of high importance in any assay procedure. Therefore, the following experiment was performed in order to repeat and confirm that the adjustments suggested to the process actually did improve the process developed so far. Some of the data would also be compared to the original process data.

**Table 3.6.** Target proteins used in experiment detailed in section 3.5 and 3.6.

| Target Protein ID | Concentration Used ($\mu$g/ml) |
|---|---|
| Cy3 Anti-Human IgG | 33.33 |
| Cy3 Anti-Human IgM | 33.33 |
| FITC Anti-Mouse IgG | 66.67 |
| FITC Anti-Mouse IgM | 16.67 |
| Cy3 rProtein-L | 10.00 |

The probes described in **Table 3.5** plus PBS were printed as described in the figure legends. The target solutions were as detailed in **Table 3.6**. In order to allow a comparison of procedures Slide Processing Method A to C, values from the PBS spots were used to calculate all the S/N values.

To demonstrate that the new process (SPM C) gives the desired results and reproducibility, the data is presented in **Figures 3.8a-e.** The reactions of the probes with target anti-human IgG are shown in **Figure 3.8a,** which shows good S/N values

with specific probes and the previously experienced cross-reactivity with rabbit and sheep IgG. **Figure 3.8b** shows good S/N values and cross-reactivity, which is higher than S/N values from specific probes. This was seen before so was not introduced by the change in procedure. The reactions of both anti-mouse target reagents is quite similar to before **(Figure 3.8c** and **Figure 3.8d),** but with some increased S/N values. The anti-mouse reagents require optimisation in terms of concentration before they will give optimal reactions.

**Figure 3.8e** shows the results obtained when Cy3 rProtein-L was added as target. This reagent had been used before and although it gave increased reactions with human serum, the S/N values were very low. Improved reactions are seen this in this experiment, with specific interactions demonstrated with monoclonal human IgG (ESD1), human serum and mouse IgG and the S/N values are also increased. With optimisation the Cy3 rProtein-L reagent could be used for quality control purposes.

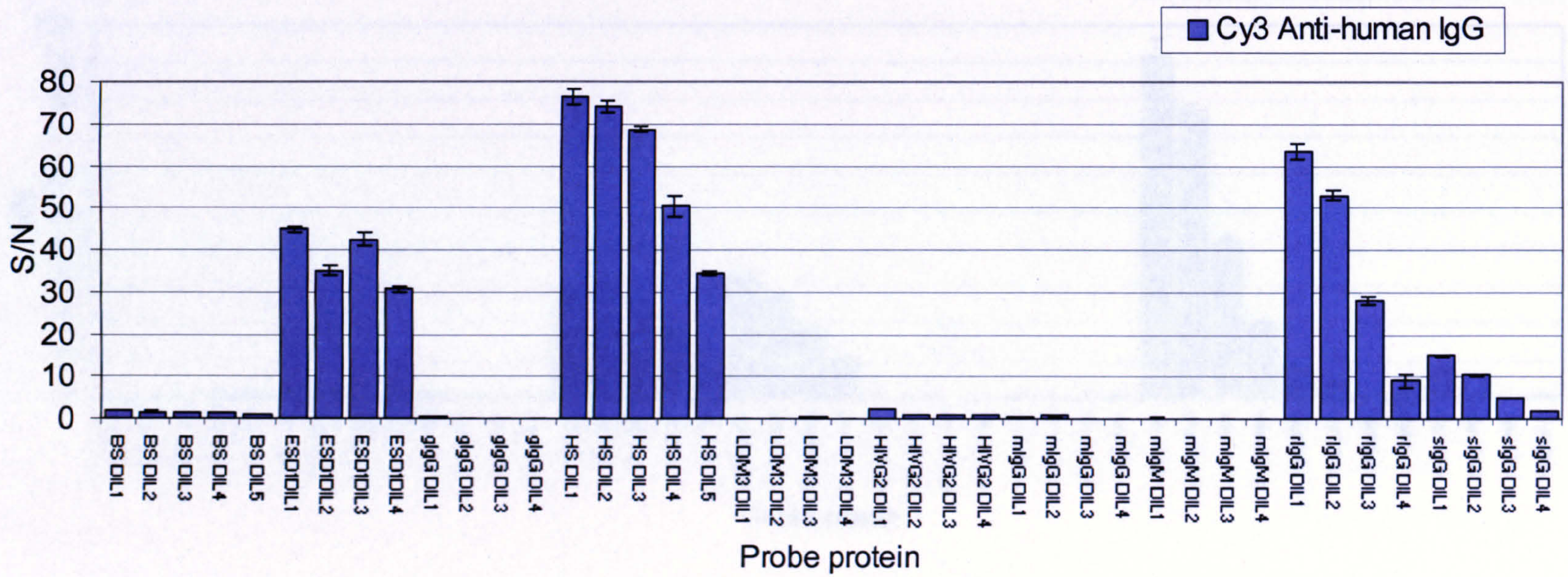



**Figure 3.8a.** Reactions of Cy3 anti-human IgG with various probe proteins, to confirm improvements in the microarray processing procedures. Slide type poly-L-lysine, slide reps 1, pins 200 $\mu$m, probes Table 3.5, probe reps 8, SPM C; blocker PBS-milk, target/volume: Cy3 anti-hIgG/25 $\mu$l, incubation 120 min, static, scanning method A.

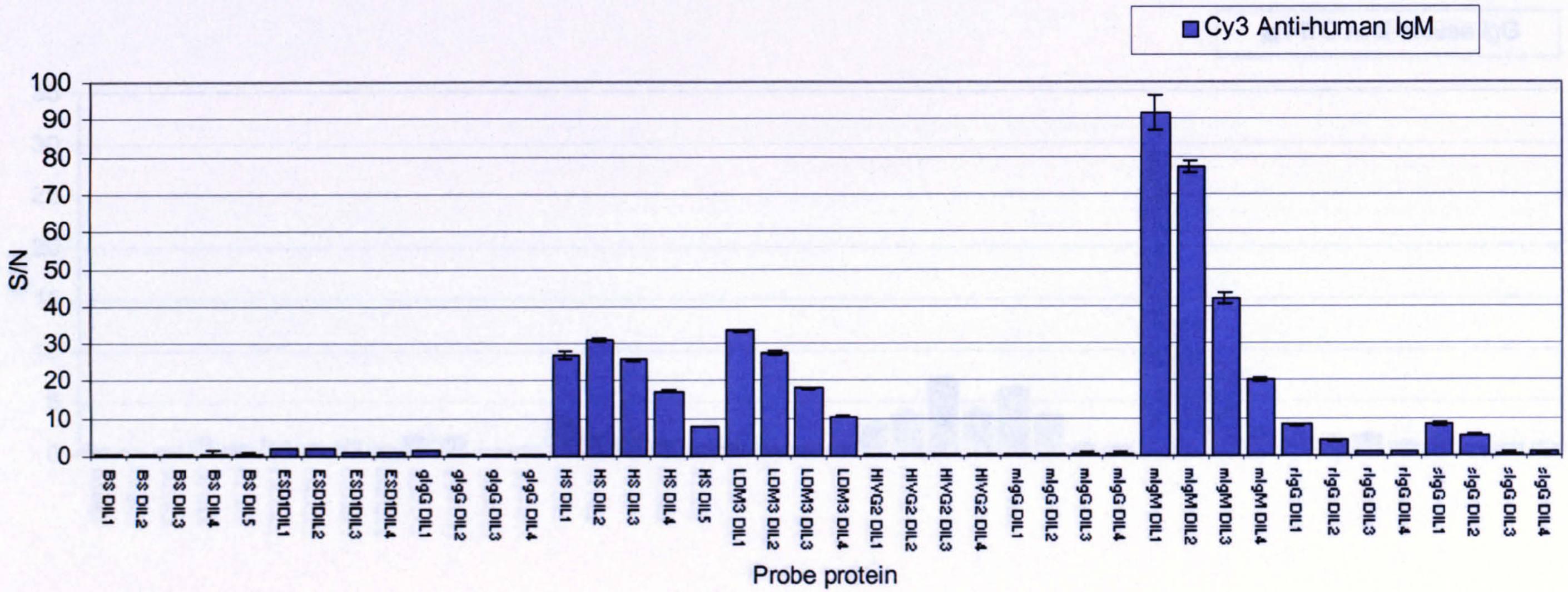



**Figure 3.8b.** Reactions of Cy3 anti-human IgM with various probe proteins, to confirm improvements in the microarray processing procedures. Slide type poly-L-lysine, slide reps 1, pins 200 $\mu$m, probes Table 3.5, probe reps 8, SPM C; blocker PBS-milk, target/volume: Cy3 anti-hIgM/25 $\mu$l, incubation 120 min, static, scanning method A.

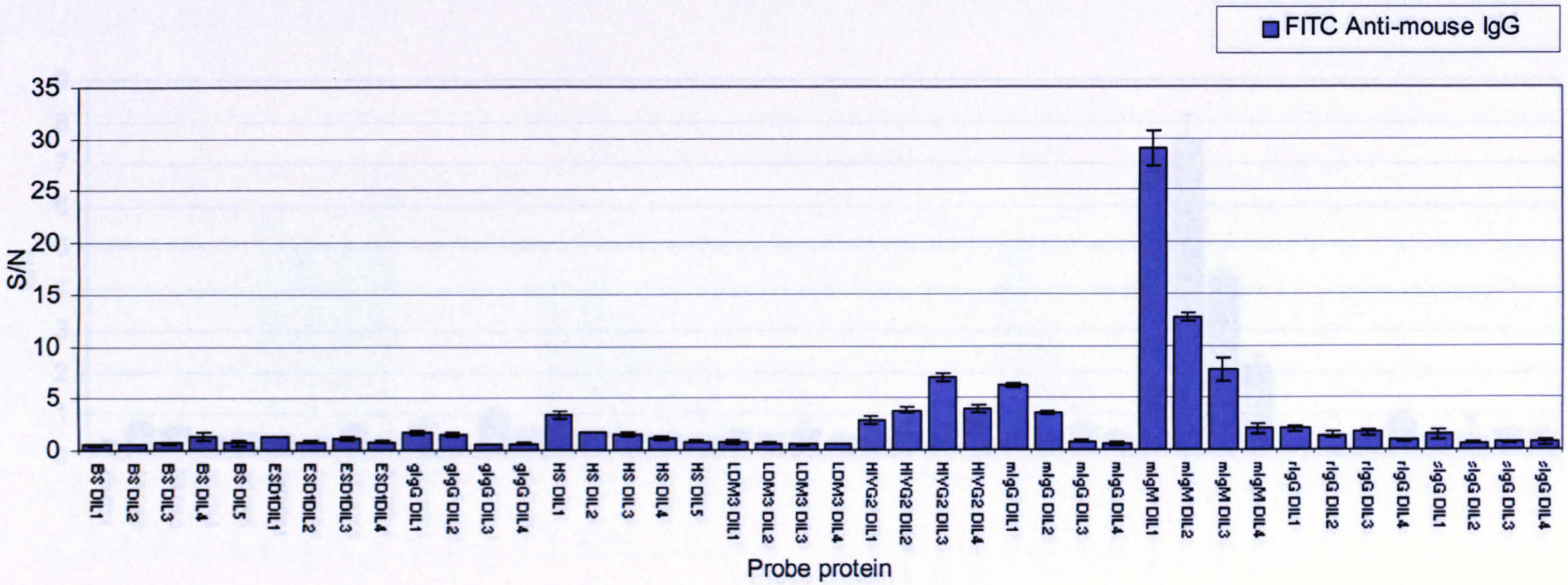



**Figure 3.8c.** Reactions of FITC anti-mouse IgG with various probe proteins, to confirm improvements in the microarray processing procedures. Slide type poly-L-lysine, slide reps 1, pins 200 $\mu$m, probes Table 3.5, probe reps 8, SPM C; blocker PBS-milk, target/volume: FITC anti-mIgG/25 $\mu$l, incubation 120 min, static, scanning method A.

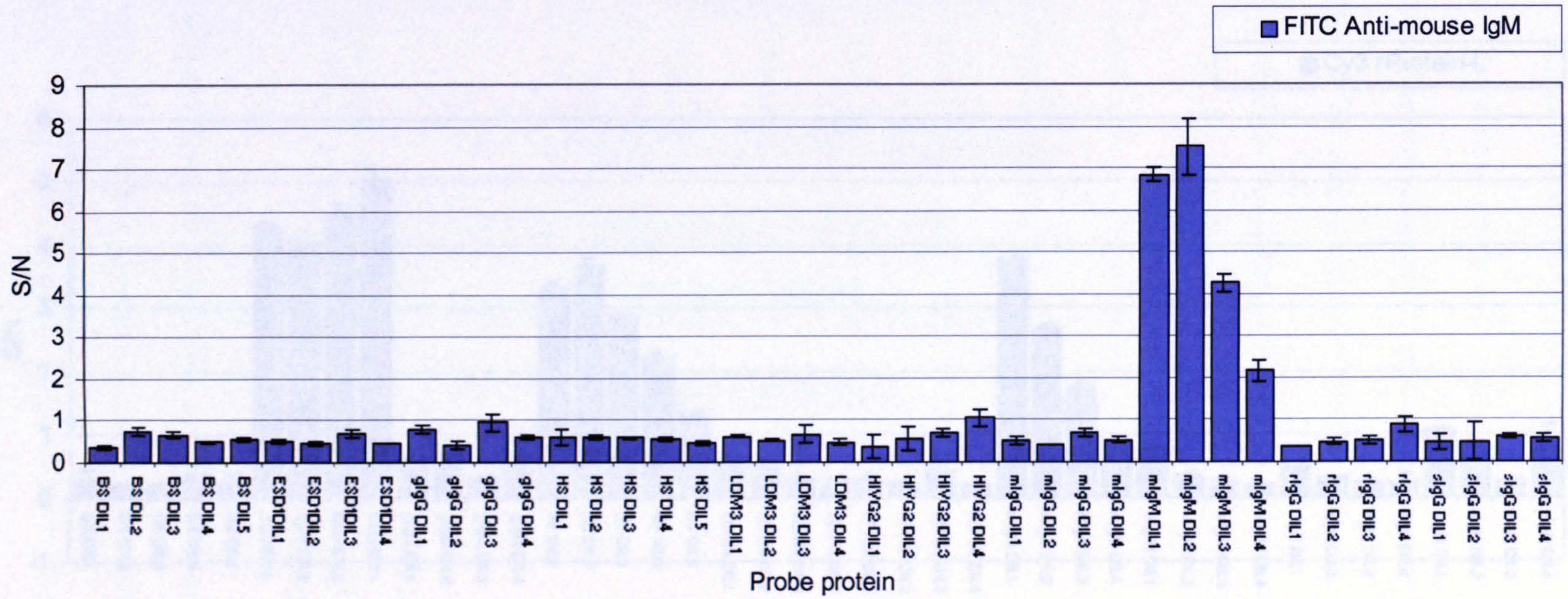



**Figure 3.8d.** Reactions of FITC anti-mouse IgM with various probe proteins, to confirm improvements in the microarray processing procedures. Slide type poly-L-lysine, slide reps 1, pins 200 $\mu$m, probes Table 3.5, probe reps 8, SPM C; blocker PBS-milk, target/volume: FITC anti-mIgM/25 $\mu$l, incubation 120 min, static, scanning method A.

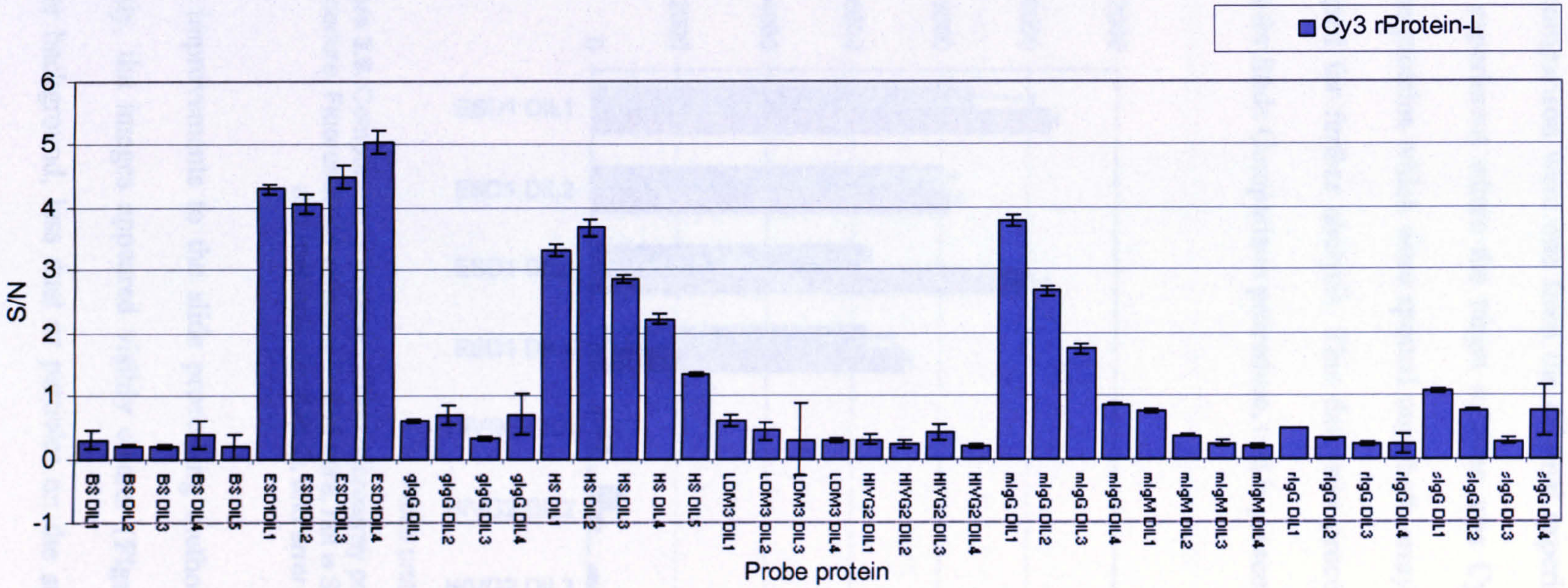



**Figure 3.8e.** Reactions of Cy3 rProtein-L with various probe proteins, to confirm improvements in the microarray processing procedures. Slide type poly-L-lysine, slide reps 1, pins 200 $\mu$m, probes Table 3.5, probe reps 8, SPM C; blocker PBS-milk, target/volume: Cy3 rProtein-L/25 $\mu$l, incubation 120 min, static, scanning method A.

To compare the original protein microarray processing procedure (SPM A) to the optimised process (SPM C), the data from two slides was compared. Those selected for comparison were one from the original experiment (section 3.2) and one from this experiment where the target solution was Cy3 anti-human IgG at 33 $\mu$g/ml. Probe proteins which were spotted onto both arrays at the same concentration were selected for further analysis. This data was processed using the Normalisation of Data for Slide Comparison procedure, and is presented in **Figure 3.9**.

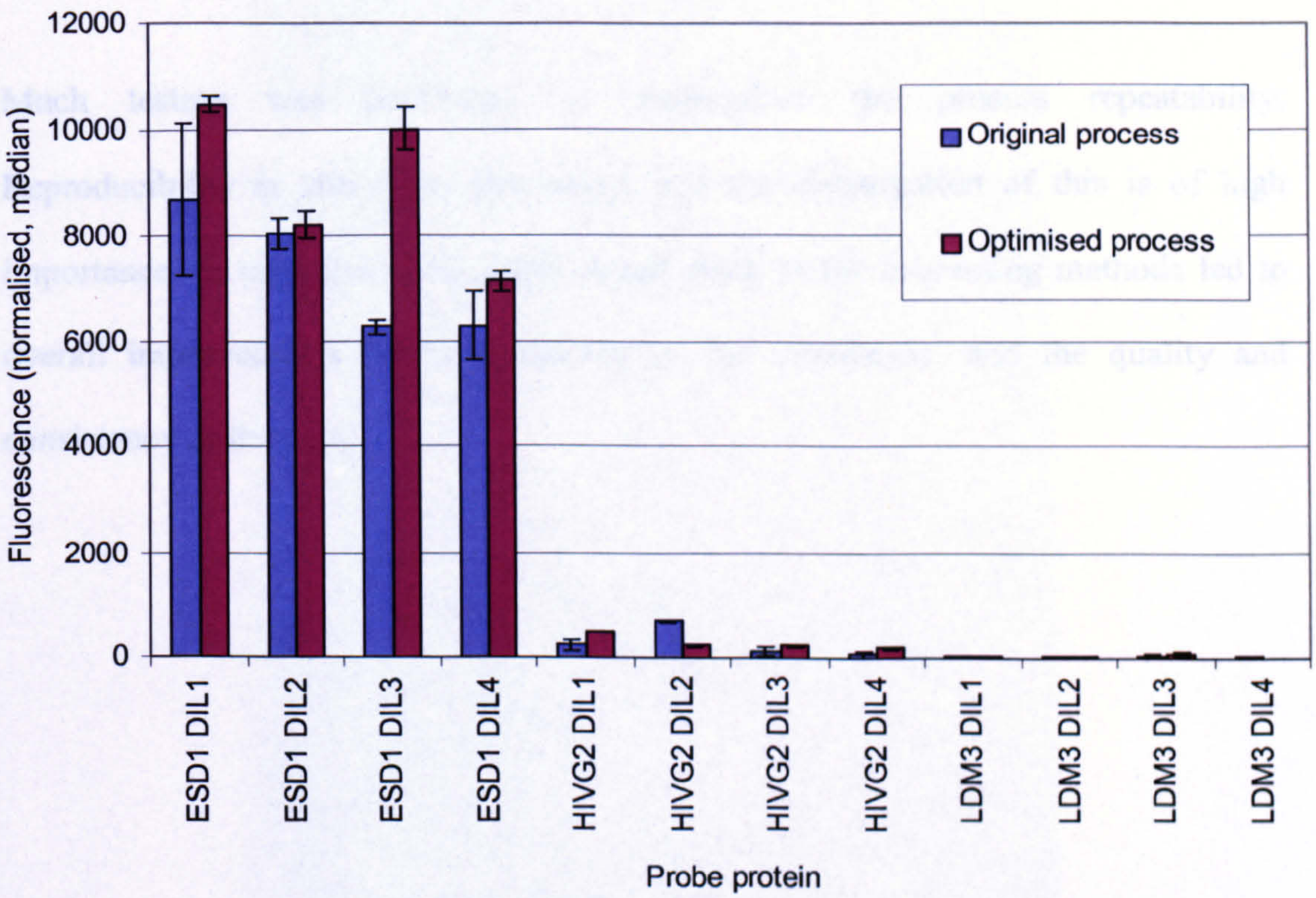



**Figure 3.9.** Comparison of original protein microarray processing procedure to the optimised procedure. Fluorescence values are used here, not a S/N ratio (normalised between slides and medians calculated), and error bars appended.

The improvements to the slide processing methods can be seen in several ways. Firstly, the images appeared visibly clearer (**Figure 3.10** and **Figure 3.11**), with lower background, less dust or particles on the surface, fewer smears, doughnuts,

black holes and comet tailing.

Secondly, once the data was analysed, increased fluorescence values were obtained. The error bars in **Figure 3.9** demonstrate the variability between replicate spots on the same slide. When the new process was used the variability is also reduced, showing that more consistency is achieved. A suitable data processing and analysis method has also been established using recommended statistical methods and using the knowledge that suitable controls with characterised reactivity must be included.

Much testing was performed to demonstrate the process repeatability. Reproducibility is critical to any assay, and the investigation of this is of high importance. In conclusion, the adjustments made to the processing methods led to overall improvements in the sensitivity of the microarray, and the quality and consistency of the data.

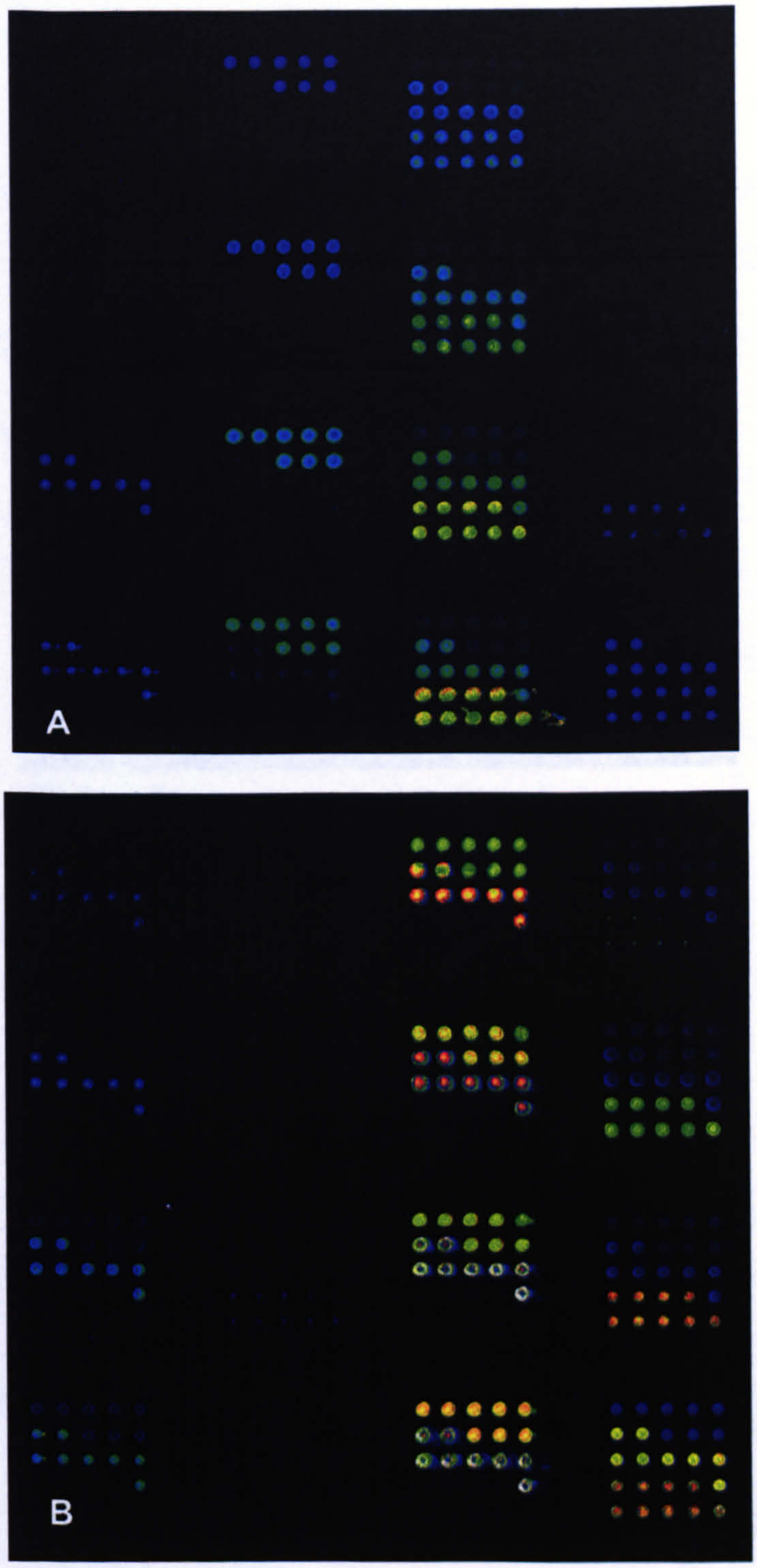
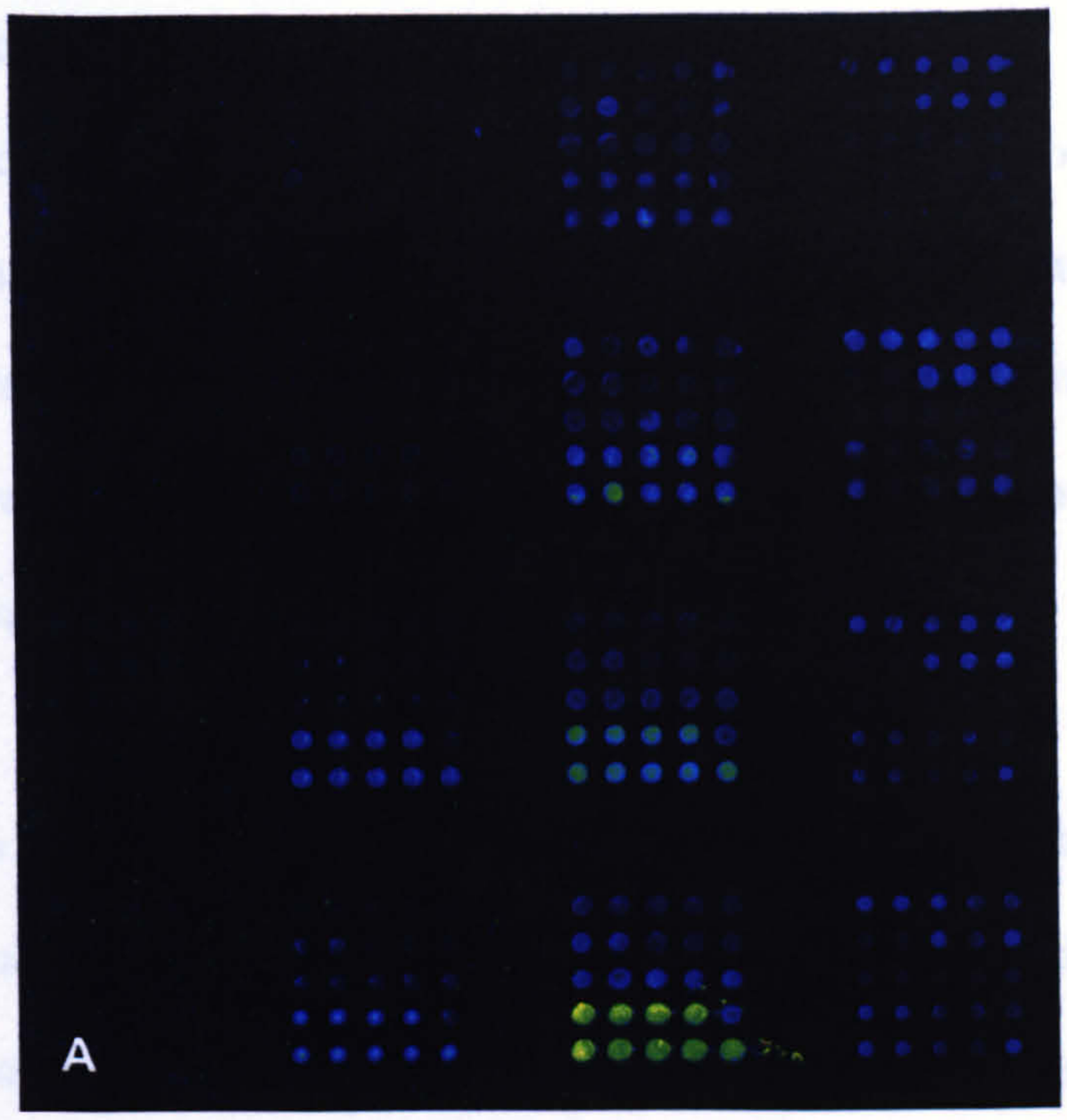



**Figure 3.10.** Pseudo colour images (blue to white scale) from slides scanned following optimisation. Slide type poly-L-lysine, slide reps 1, pins 200 $\mu$m, probes Table 3.5, probe reps 8, SPM C; blocker PBS-milk, target/volume: A=Cy3 anti-hIgM, B= Cy3 anti-hIgG/25 $\mu$l, incubation 120 min, static, scanning method A.



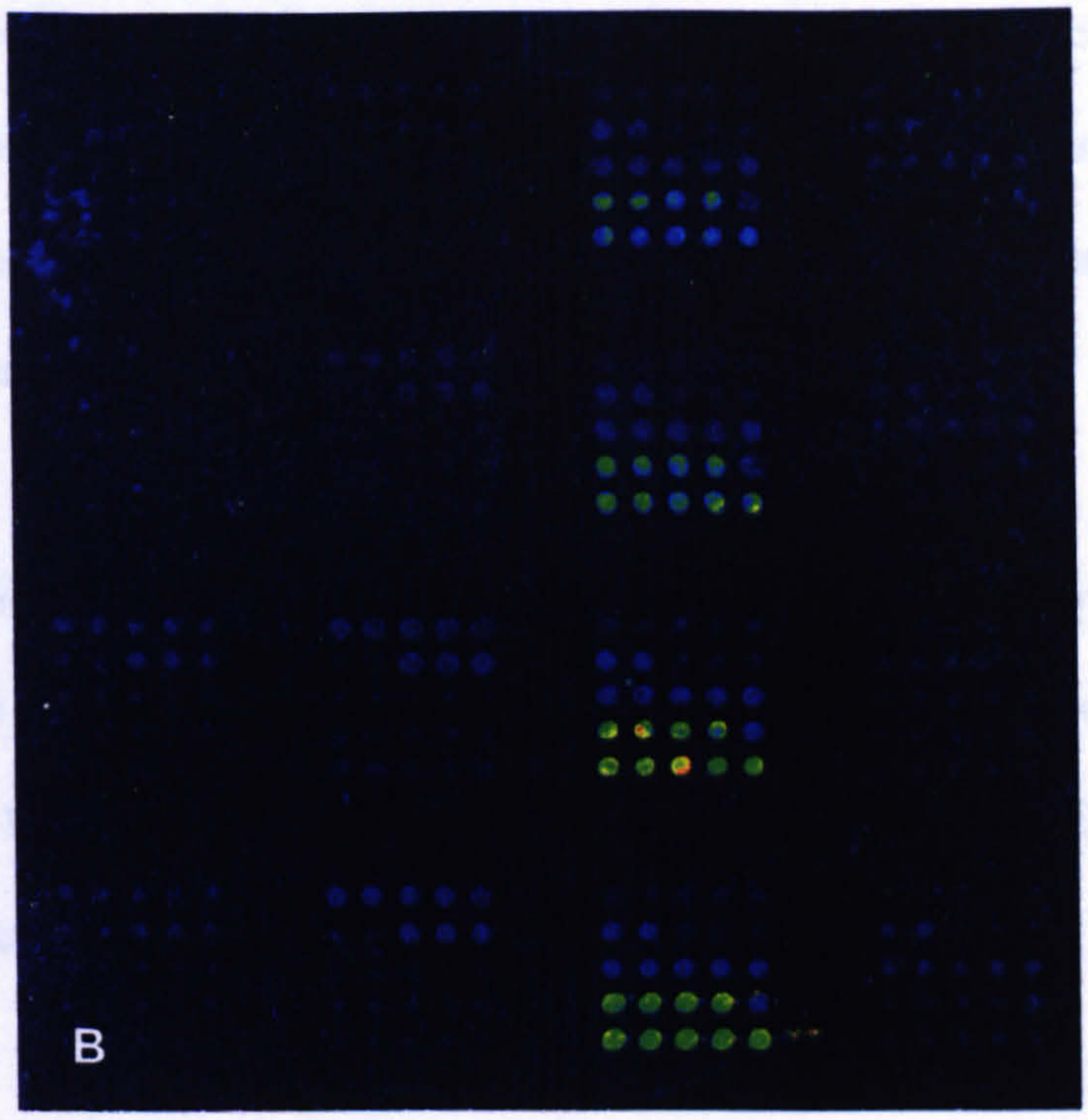




**Figure 3.11.** Pseudo colour images (blue to white scale) from slides scanned following optimisation. Slide type poly-L-lysine, slide reps 1, pins 200 $\mu$m, probes Table 3.5, probe reps 8, SPM C; blocker PBS-milk, target/volume: A=FITC anti-mIgG B= FITC anti-mIgM/25 $\mu$l, incubation 120 min, static, scanning method A.

## 3.5 Comparison of Different Slide Surface Chemistries

Poly-L-lysine coated slides were used in this work to establish protein microarray processing methods and capabilities. These slides were relatively cheap and easy to prepare and gave a platform, from which much useful data was obtained. Other slide types were available with varying surface chemistries, but when purchased commercially were prohibitively expensive.

In an attempt to further optimise the protein microarray procedure, a range of slides with varying surface chemistries were assessed using the standardised processing methods (with one exception in which the manufacturers recommended procedures were supplied with specific reagents for use with the slides). The slide surfaces investigated are detailed in **Table 3.7.** Proteins may be affected by their method of attachment to a solid support, and may give higher or lower binding to ligand depending on the conditions.

The probes described in **Table 3.5** were printed onto poly-L-lysine, amino silane, polyacrylamide and nitrocellulose slides. Additional spots of the spotting buffer, either PBS or 1X Array Buffer, (spotting buffers as described in Chapter 2) were spotted onto these microarrays for use in data analysis.

**Table 3.7** Slide substrates used in experiment in 3.5 and details of binding methods.

| Slide Surface Coating | Description | Binding action |
|---|---|---|
| Poly-L-lysine | High MW compound with free amino groups | Positive charge at neutral pH. Binds negatively charged proteins through electrostatic forces |
| Polyacrylamide | Cross-linked matrix that holds molecules in 3-D | Traps molecules in matrix |
| Amino Silane | Two reactive groups bound to silicon atom in the molecule. Hydroxy group at one end binds to glass, amino group at other end | Binds negatively charged proteins through electrostatic forces |
| Nitrocellulose | Nitrocellulose polymer. | Positive charge binds negatively charged proteins by electrostatic forces |
| Streptavidin | Amine groups of proteins are biotinylated using biotinyl-N-hydroxysuccinimide ester | Biotin has high affinity for the four tryptophan residues on each streptavidin unit |

**Table 3.8.** Biotinylated antibodies spotted on streptavidin coated slide, and concentration in $\mu$g/ml.

| Antibody ID | Antibody type | Conc. DIL1 | Conc. DIL2 | Conc. DIL3 | Conc. DIL4 |
|---|---|---|---|---|---|
| LA2 | mouse IgM | 55 | 37 | 18 | 14 |
| ES9 | mouse IgM | 81 | 54 | 27 | 20 |
| LB2 | mouse IgM | 108 | 72 | 36 | 27 |
| ES15 | mouse IgM | 127 | 85 | 42 | 32 |
| LDM3 | human IgM | 6.4 | 3.2 | 1.6 | 0.8 |
| ESD1 | human IgG | 300 | 200 | 100 | 75 |
| HIVG2 | mouse IgG | 300 | 200 | 100 | 75 |

Streptavidin coated slides were printed and investigated independently, due to probe concentration differences to the non-biotinylated probes. The probes were biotinylated antibodies described in **Table 3.8,** which should bind to the streptavidin on the slide surface.

Once processed, the values from the spotting buffers were used to calculate noise and the results are presented in **Figures 3.12a-c.** The data in **Figure 3.12a** showed that specific interactions between probe proteins and Cy3 anti-human IgG consistently gave higher S/N values on the polyacrylamide surface compared to all other surfaces. The good performance of the polyacrylamide surface may be due to several reasons, which are discussed at the end of the chapter.

The data shown in **Figure 3.12b** shows the reactions of expected negative probes, showing a level of cross-reactivity with rabbit IgG that has been evident before. Although levels of positive S/N are higher on polyacrylamide, levels of NSB are not increased. The values of an expected positive probe (ESD1 DIL1) are divided by the negative probe values to give a ratio on the different slide surfaces.

In **Table 3.9** the positive/negative probe values are shown, the higher the value then the lower the NSB for each probe on each surface. This data shows that the polyacrylamide surface consistently gives higher ratios, therefore lower background/NSB.

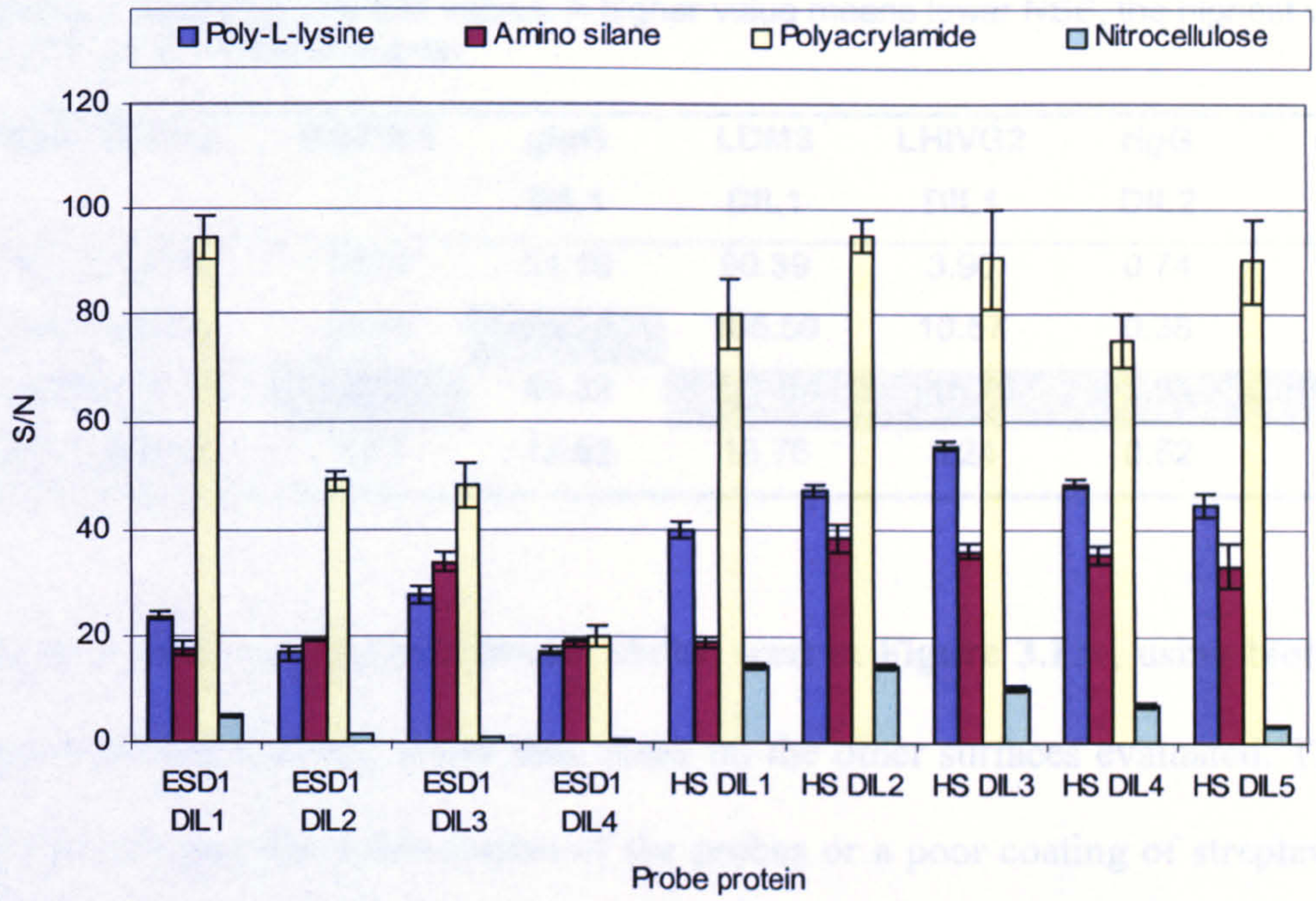



**Figure 3.12a.** Reactions of Cy3 anti-human IgG against human IgG containing probe proteins on different surfaces. Slide type various, slide reps 1, pins 200 $\mu$m, probes Table 3.5, probe reps 6, SPM A; blocker PBS-milk, target/volume: Cy3 anti-hIgG/25 $\mu$l, incubation 120 min, static, scanning method A.

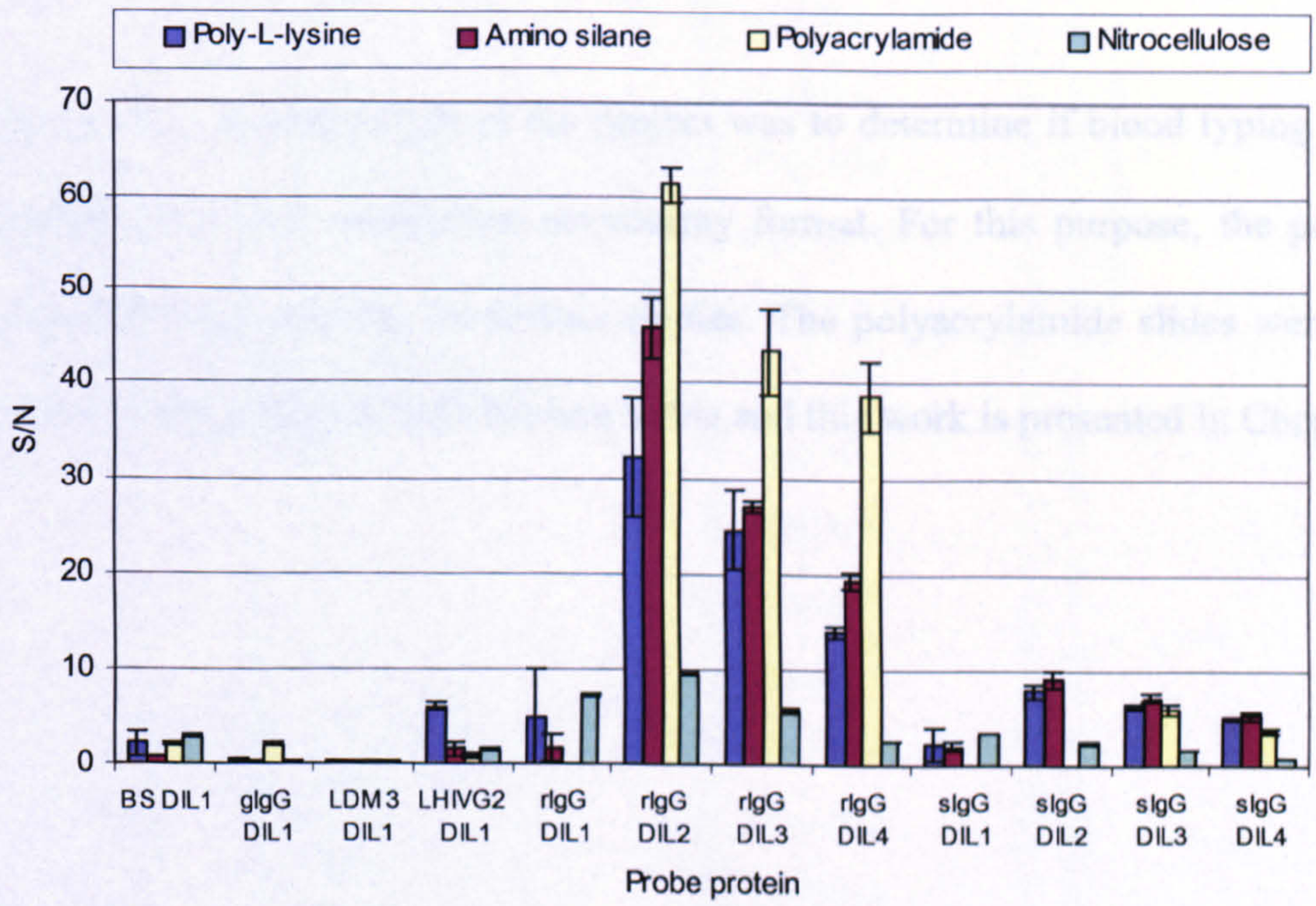



**Figure 3.12b.** Reactions of Cy3 anti-human IgG against various probe proteins on different surfaces, showing cross-reactivity levels. Slide type various, slide reps 1, pins 200 $\mu$m, probes Table 3.5, probe reps 6, SPM A; blocker PBS-milk, target/volume: Cy3 anti-hIgG/25 $\mu$l, incubation 120 min, static, scanning method A.

**Table 3.9.** Results of expected positive probe S/N value, from ESD1 DIL1, divided by expected negative probe S/N values. A higher value means lower NSB, the highest value for each probe is indicated in grey.

| Slide surface | BS DIL1 | gIgG DIL1 | LDM3 DIL1 | LHIVG2 DIL1 | rIgG DIL2 | sIgG DIL1 |
|---|---|---|---|---|---|---|
| Poly-L-lysine | 10.97 | 54.16 | 90.39 | 3.90 | 0.74 | 10.59 |
| Amino silane | 23.04 | 75.10 | 105.59 | 10.57 | 0.38 | 8.49 |
| Polyacrylamide | 43.30 | 45.32 | 277.64 | 115.76 | 1.54 | 1957.18 |
| Nitrocellulose | 1.71 | 12.62 | 18.76 | 3.24 | 0.52 | 1.42 |

The S/N values on the streptavidin slides, seen in **Figure 3.12c**, using biotinylated probes are substantially lower than those on the other surfaces evaluated. This may be due to either low biotinylation of the probes or a poor coating of streptavidin on the slides. As the biotinylation of probes is an extra step in the process, shown here not to improve results substantially, the streptavidin slides were not investigated further.

Following this, the next stage of the project was to determine if blood typing could be performed on this established microarray format. For this purpose, the poly-L-lysine slides were selected for further studies. The polyacrylamide slides were also selected due their superior S/N binding ratios and this work is presented in Chapter 4.

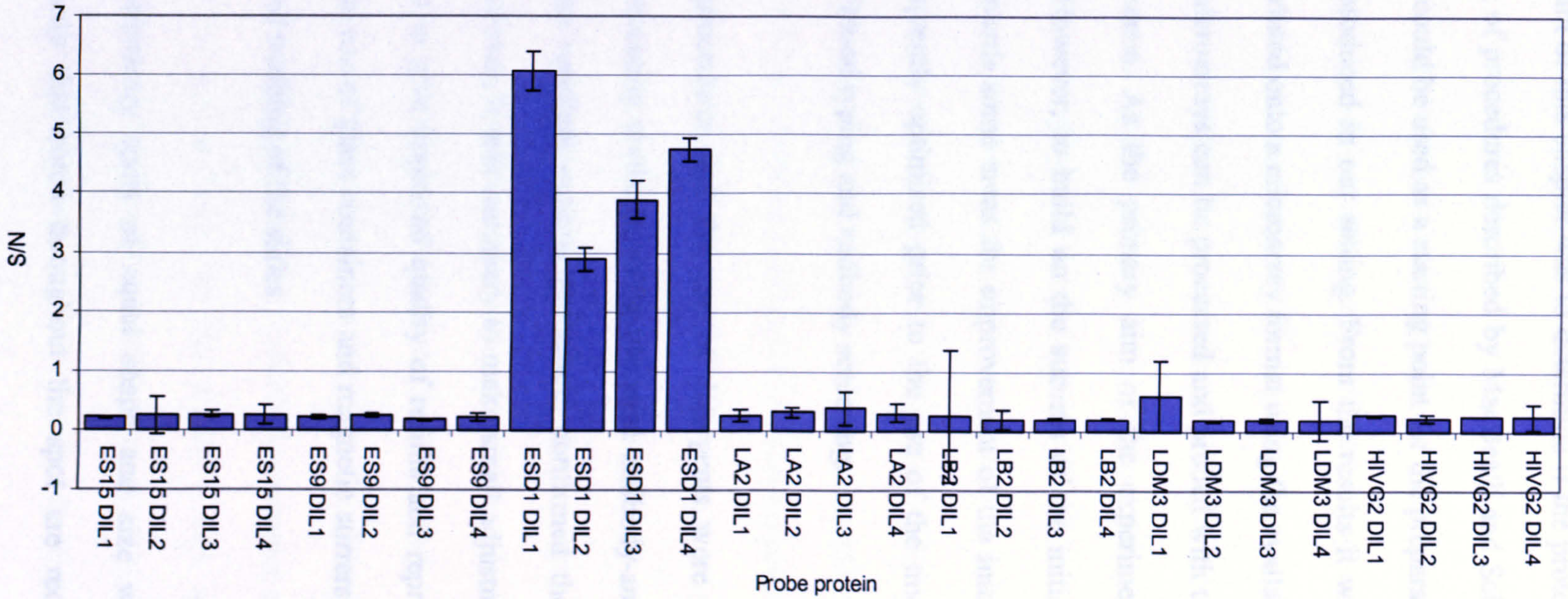



**Figure 3.12c.** Reactions of Cy3 anti-human IgG against various biotinylated antibody probes on a streptavidin coated slide surface. Slide type streptavidin, slide reps 1, pins 200 $\mu$m, probes Table 3.8, probe reps 6, SPM A; blocker PBS-milk, target/volume: Cy3 anti-hIgG/25 $\mu$l, incubation 120 min, static, scanning method A.

## 3.6 Chapter Discussion and Conclusions

### 3.6.1 Chapter Discussion

The first aim of this chapter was to determine if the protein microarray printing and processing of procedures described by MacBeath and Schreiber (2000) and Haab *et al.* (2001) could be used as a starting point for the preparation of protein microarrays and be reproduced in our setting. From the results it was concluded that proteins could be printed onto a microarray format using the available equipment, and that the resultant microarrays can be processed and probed with target proteins to determine probe presence. As the primary aim of the experiment, this was a successful outcome. However, to build on the success of the initial experiments, the results pointed towards some areas for improvement of the initial procedure. The process was subsequently optimised prior to the use of the microarrays for the intended purpose of blood typing and antibody screening.

Blocking procedures and the use of detergents were investigated to determine optimal processing methods, using classical antibody-antigen sets of proteins and ligands. The resultant optimal procedure, confirmed the findings of Haab *et al.* (2001). However, it was necessary to make small adjustments to different stages of the process to give improved quality of results and reproducibility. Improvements included the use of glass containers and magnetic stirrers with customised racks for blocking and washing of the slides.

Ideally, microarray spots of equal shape and size with immobilised material homogenously distributed throughout the spot are required. Conditions during

spotting may affect spot quality, producing imperfect spots including those described as doughnuts or small spots. Conditions during processing can influence the data quality and result in comet tailing, drying out or spot removal.

Humidity during printing was introduced to reduce doughnut shaped spots. A re-hydration step prior to blocking was included to reduce the complete removal of probe spots during incubation with targets. Comet tailing was minimised with improvements to the processing and can be affected by blocking. To avoid drying of fluorescent target onto the slides, the incubation was performed in a purpose built chamber with an underlayer to reduce loss of moisture. All process improvements were included in the optimised procedure, referred to as Slide Processing Method C. When the improved process was compared to the original process, several improvements were seen. Even small improvements may mean the difference between detection of weak positive reactions and negatives.

The use of target anti-human IgG was selected to perform most optimisation experiments, mainly due to the high level of specificity and reactivity shown with human serum and human IgG antibodies. Other detection/target antibodies used either gave lower S/N or showed greater cross-reactivity. The cross-reactivity found using certain reagents was to be expected as the antibodies were from polyclonal source and had not been adsorbed to remove unwanted antibodies. Cross reactivity may also be due to many of the antibody cell lines being human-mouse heterohybridomas. The experiments using various anti-species antibodies reinforced the importance of selecting the best reagents for the purpose, to achieve high

specificity and signal. If these reagents were to be used further, they would require anti-species adsorption. This would involve testing titre pre and post adsorption and tests by saline and papain spin tube with group A, B and O cells, as animal sera may also contain anti-A or anti-B. The results obtained using anti-species reagents showed that concentration of the anti-mouse IgG and anti-mouse IgM would require adjustment to give enhanced reactions, but it is unlikely that a change in concentration would increase or decrease cross reactivity; efforts can only be made to reduce NSB. This is similar to results found in Haab *et al.* (2001). They reported that a variation in fluorescent target labelling is a prominent source of variability in fluorescence measurements. As well as variability in the concentration of the target antibodies, there may also be variability in the number of fluorescent molecules attached to each antibody. If required, variability could be rectified for subsequent analysis by preparing titrations of target antibodies against probes to establish equal levels of antibody and fluorescence, or the labelling procedure could be modified to allow greater comparison between slides. This was not required for this work, as only a demonstration of presence of the probes was required. In some results (specifically in **Figures 3.3a, e, f and l)** the unexpected reactions may be contributed to the 'hook' effect (Fernando and Wilson, 1992). The hook effect occurs mostly in one step sandwich immunoassays, and is characterised as a decrease in assay response occurring at high analyte concentrations. This is one possible explanation for lower values at highest concentrations.

During this stage of the project a procedure to analyse the data was developed (detailed in Chapter 2). To summarise the data processing method, it involves the

initial determination of data quality before further processing, then the subtraction of background for each spot and analysis to determine the 'best' PMT scan. This is the scan that has used high enough excitation to allow the detection of low or weak signal, yet not too high to give saturated spots that in turn give unrepresentative values. Following this the selected negative control spots are used to calculate a mean plus two standard deviations, equal to the background noise, and this is used to calculate a signal to noise (S/N) ratio for each spot. Following this, a median of replicate spots was calculated. Error bars were included where appropriate and indicate the variability obtained from replicate probe spots on the array. The method of analysis was retrospectively applied to all data represented in this thesis. Initially, S/N ratio was performed using the actual mean of the negative control spots. Considerations in data analysis were reviewed with the assistance and direction of a statistician, Thorsten Forster of the SCGTI, who recommended the use of noise (or background) as the mean plus two standard deviations of the negative spots. This is a recognised statistical method used to provide signal ratios.

An appropriate biological negative control is an antibody similar in structure and/or species to the expected positive probe, but which is directed to an unrelated or simply different antigen not present in the target solution. However, it is important that the selected control probes do not demonstrate a high level of non-specific binding or cross-reactivity. For example, if the control probe demonstrates a high level of non-specific binding then the test probes are likely to give reduced S/N values. This was demonstrated in section 3.3.2 (Further Optimisation of Blocking Procedure Using Detergents) where the data was processed in two ways, using different probes as

negative controls to calculate noise. As some of the negative control probes actually showed cross-reactivity, it indicated that negative probes should be observed for expected reactions before using as background noise and that controls must be selected carefully. The reactivity of all probes and target materials should be well characterised or results may be lost or unrepresentative.

When a comparison of four different slide surfaces was performed using the classical antibody-antigen type reaction, the polyacrylamide slides were superior to all other types (refer to **Table 3.9**). The matrix of the polyacrylamide gel coating gives a 3-D support for proteins, and due to its depth may allow for an increased capture of probe antibody, leading to the higher level of subsequent binding of the target protein. The nature of the matrix may mean that less probe and target are washed off the slide in the various stages of the process. The nitrocellulose surface was least suited to the interactions used for evaluation. While both poly-L-lysine and amino silane slides showed similar reactivity, the poly-L-lysine slides provided a satisfactory and cost efficient matrix for the antibody-antigen interactions, and can be prepared in-house. Therefore, polyacrylamide and poly-L-lysine slides were selected for further evaluation in Chapter 4, which uses the work in this chapter as a basis to develop a blood typing microarray platform.

### 3.6.2 Chapter Conclusions

The aims of this stage of the project have been met as follows:

- *To determine the ability to fabricate protein microarrays using available equipment and using publications detailing similar type studies, probe the microarrays with anti-species detection/target solutions to determine if probes are retained on the slides.*

  Protein microarrays have been printed and processed and subsequent experiments have shown that protein probe–ligand reactions can be reproducibly detected on the slides.

- *To optimise protein microarray procedures such as blocking, washing, incubation conditions, detection methods, data extraction and data processing methods, to enable the study of probe-ligand interactions.*

  Processing procedures have been examined and optimised resulting in higher S/N ratios for probe – ligand interactions and less variance in replicates. Two types of fluorophores have been used with success, and data analysis methods have been adapted to suitably analyse each data set.

- *To evaluate different slide surface chemistries and select options for the next stage of the project, the development of blood typing microarrays.*

  Various surfaces have been evaluated and two selected for further study in the investigation of probe-ligand interactions for the purpose of multi-parameter blood typing.

- *Amalgamate all findings and process optimisations to provide a basis for a multi-parameter blood typing microarray platform.*

Protein microarray procedures have been optimised using sets of proteins and ligands, providing a solid basis for blood typing microarrays.

The conclusions drawn from the work in this chapter will be applied in the next chapter, which describes probe-ligand interactions on a microarray format for use in blood typing.

# CHAPTER 4

# NON-AGGLUTINATION MULTI-PARAMETER BLOOD TYPING IN A MICROARRAY FORMAT

# 4. NON-AGGLUTINATION MULTI-PARAMETER BLOOD TYPING IN A MICROARRAY FORMAT

## 4.1 Introduction and Chapter Aims

Chapter 3 gave the basis of a solid-phase microarray platform for the study of probe-ligand interactions and provided important information on aspects of fabrication, processing and detection of probe-ligand interactions using a microarray format. The next step was to determine whether the probes retained functionality. This chapter addresses this, and extends the work to develop a 'dual' solid-phase system for the purpose of blood typing, where the immobilised probes are blood typing antibodies and the targets are blood group antigens carried on the surface of a larger particle, in this case erythrocytes. As the antigens are carried on the erythrocyte, they too can be considered in 'solid-phase' as they are effectively immobilised to a solid support.

Solid-phase blood typing interactions can be detected by methods other than haemagglutination. As the probes are not in liquid-phase, haemagglutination does not arise, and the interaction must be detected by an alternative method. For instance, if an antibody is immobilised and binds an erythrocyte, detection of the interaction can be through direct or indirect labelling as illustrated in **Figure 4.1.** This chapter looks at several techniques for detection of the antibody-erythrocyte interaction. If the membrane of the erythrocyte is labelled, then the detection is considered direct. If a label were attached via another molecule (e.g. an antibody or lectin) then this would be considered primary indirect labelling. If a labelled reagent is added once the cells are already bound to the array, then for the purpose of this thesis is considered secondary indirect labelling

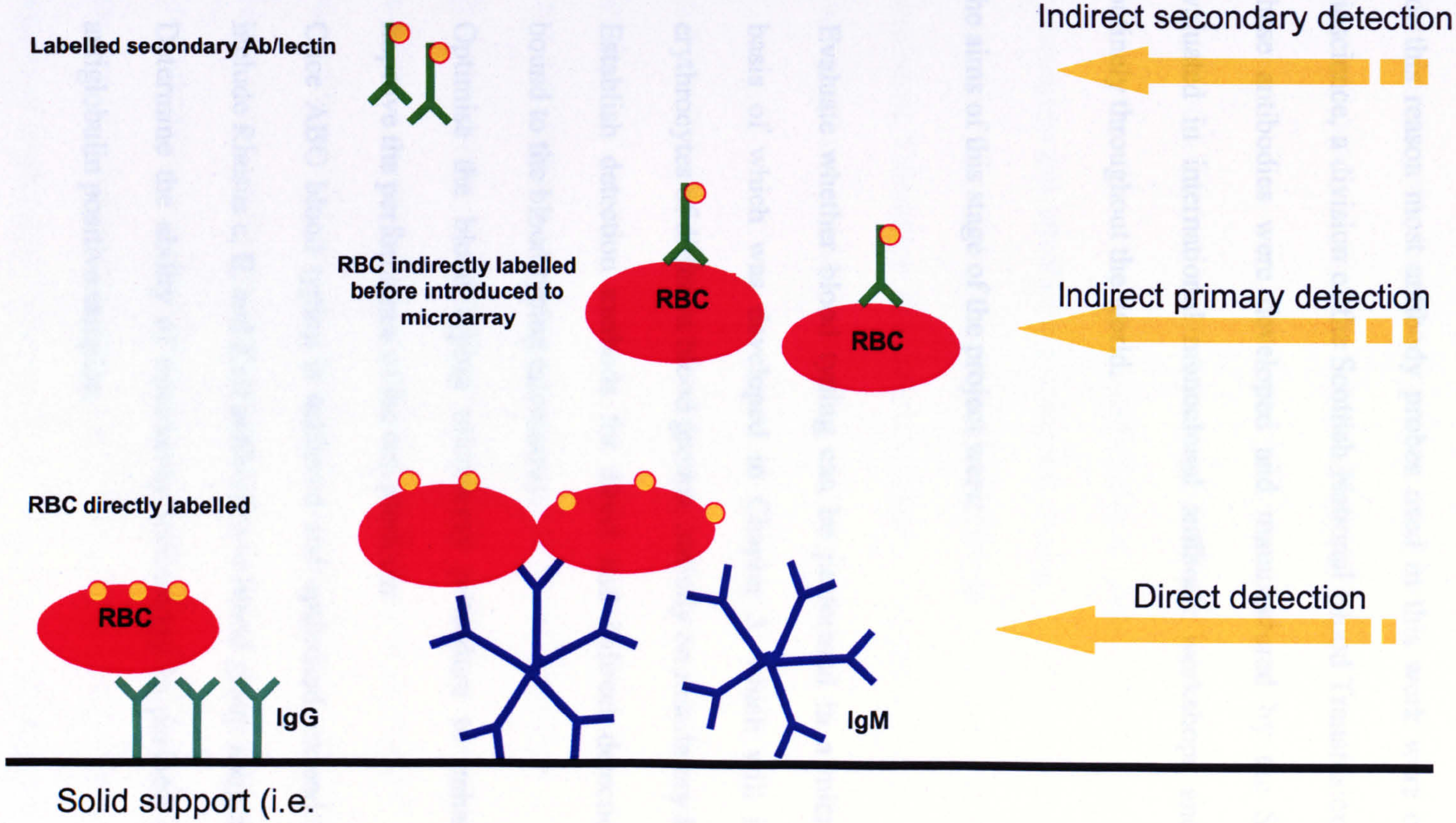



**Figure 4.1**. Schematic of non-agglutination based solid-phase blood typing and direct, indirect primary or secondary detection of the Ab-erythrocyte interaction, as proposed in this thesis.

The selection of Ab probes for the purpose of routine blood typing is of critical importance. Legislation in blood typing states reagents must be well characterised and assessed in performance evaluation field trials and in rigourous stability testing. For this reason most antibody probes used in this work were obtained from Alba Bioscience, a division of the Scottish National Blood Transfusion Service (SNBTS). These antibodies were developed and manufactured by the SNBTS, have been evaluated in international monoclonal antibody workshops and several are used routinely throughout the world.

The aims of this stage of the project were:

- Evaluate whether blood typing can be performed in a microarray format, the basis of which was developed in Chapter 3, which will involve the use of erythrocytes of different blood groups, initially on mandatory RhD and ABO.
- Establish detection methods for direct and indirect detection of erythrocytes bound to the blood typing microarray.
- Optimise the blood typing microarray procedure to enhance reactions and improve the performance of the test platform.
- Once ABO blood typing is achieved and optimised, extend the probe panel to include Rhesus c, E, and Kell antibodies to blood group antigens.
- Determine the ability of microarray technology to perform detection of direct antiglobulin positive samples.

Details of all methods and reagents used can be found in Chapter 2. The information that defines the protein microarray printing, processing characteristics and describes target solutions for each experiment, will be detailed in the figure legends. However, where the target is not fluorescently labeled and a detection reagent is added, both target and detection solutions will be described.

## 4.2 Blood Typing Interactions by Microarray

This section looks at the interactions of ABO blood group antibodies with erythrocytes and whether a microarray format could accommodate a blood typing interaction. The optimal method for detection of bound erythrocytes had to be established. Throughout the early blood typing microarray work, many methods were investigated and are detailed in the following sections.

### 4.2.1 Indirect Erythrocyte Detection Using Fluorescently Labelled ConA

#### 4.2.1.1 RhD Blood Grouping Using Indirect Detection

For the detection of erythrocytes bound to a microarray, indirect labelling methods were first investigated. An ideal detection molecule should bind to all erythrocytes irrespective of blood group. As described in Chapter 1 (1.3.2.5.3), Concanavalin A (ConA) is a lectin that binds to glycoproteins through mannose and glucose residues, in the presence of calcium and manganese ions. Therefore, ConA was considered for detection of bound erythrocytes in blood grouping microarrays in this thesis. For the purpose of erythrocyte detection, ConA could be either attached to the erythrocyte surface prior to addition to the array (indirect primary labelling), or added as a

secondary reagent (indirect secondary labelling) to attach to erythrocytes already bound to the microarray.

ConA was first evaluated to confirm that it would bind to erythrocytes. 1 mg/ml ConA solution was titrated against erythrocytes of various blood groups using the IgM Antibody Haemagglutination Assay, with cells suspended in PBS-Ca/Mn. The titration end point averaged 256, equivalent to a ConA concentration of 3.9 $\mu$g/ml, and on the basis of calculations have between 100-200K molecules per erythrocyte (see calculations in Appendix 2). This testing demonstrated that ConA binds to erythrocytes, and also demonstrated the limits for occurrence of haemagglutination.

Both FITC and Cy3 labelled ConA were used for detection. Fluorescence activated cell sorter (FACS) analysis was performed using the Analysis of Fluorescently Labelled Erythrocytes method. The results are presented in Appendix 2, **Table A2.** The results showed fluorescence is detected on erythrocytes only when the calculated level of ConA is above 100K molecules per erythrocyte when using FITC labelling, and at one million per cell if using the Cy3 labelled ConA. It may be that there are fewer Cy3 molecules per ConA incorporated during the labelling process and it was decided to only use the FITC ConA. When tested in a haemagglutination assays, 100K ConA per erythrocyte was also the level at which visible haemagglutination ceases. A higher level of ConA may not be ideal for detection as clumps of agglutinates could form and obscure the array.

Many blood grouping microarrays were attempted at this point, but when scanned no fluorescence was evident and no erythrocytes were attached to any of the arrays when examined microscopically. It may be that the ConA bound to the erythrocyte masks the cells and consequently makes the blood group antigens inaccessible to the antibody probes on the microarray. To test this theory, a suspension of the ConA labelled cells (calculated at 100 molecules per cell, see Appendix 2) was tested against the antibody probes that had been used on the microarray, in liquid phase haemagglutination assay, as described in Chapter 2. There was no agglutination present, and this confirmed that the interaction of the cells with antibody is inhibited by the presence of ConA.

To further determine if indirect primary labelling could be used, and to test the above theory, a blood typing microarray experiment using indirect primary and indirect secondary labelling was performed using ConA and anti-Rh29. Rh29 is present on all except $Rh_{null}$ phenotype erythrocytes (reviewed in Daniels, 2002). Several blood group antigen specific antibody probes and ConA and PBS were used. The erythrocytes used were group O $R_1r$ (CcDee).

For the indirect primary labelling, cells were sensitised with FITC ConA (3.9 $\mu$g/ml in PBS-Mn/Ca) or Cy3 Anti-Rh29 (at 60 $\mu$g/ml in PBS) prior to addition to the microarray. For the indirect secondary labelling, erythrocytes were added to the microarray followed by the addition of either FITC ConA or Cy3 anti-Rh29, similar to a typical sandwich type assay.

The results of this work gave no clearly specific reactions or meaningful results. It was concluded that no erythrocytes were binding to the probe antibodies, and this was confirmed by microscopic examination. This experiment used cells of Rhesus phenotype $R_1r$, which is known to have a relatively weak expression of the D antigen at between 9,900-14,600 antigen sites per cell (Rochna and Hughes-Jones, 1965; Hughes-Jones *et al*, 1971). It was decided to test cells with a higher antigen density per cell.

#### 4.2.1.2 ABO Blood Grouping Using Indirect Detection

In 4.2.1.1 two monoclonal antibodies against the blood group RhD antigen (one IgG and one IgM) failed to bind RhD positive erythrocytes. Therefore, erythrocytes expressing blood group B were selected. IgM mouse monoclonal antibody cell line LB2 is directed against blood group B antigen, and gives strong agglutination when tested in liquid phase haemagglutination assays. This antibody was spotted along with other probes as described in **Table 4.1.** The two target solutions were FITC ConA and Cy3 Anti-Rh29.

This experiment gave no meaningful results from either the control slides or those to which erythrocytes and target solution were added. Both the probe antibodies and the detection molecules bind erythrocytes in liquid phase assays (data not shown).

**Table 4.1.** Probes spotted for experiment in 4.2.1.2, and concentration in $\mu$g/ml.

| Probe/ Antibody ID | Protein/ Ab type | Conc. DIL1 | Conc. DIL2 | Conc. DIL3 | Conc. DIL4 |
|---|---|---|---|---|---|
| Anti-A (LA2) | mouse IgM | 55 | 37 | 18 | 14 |
| Anti-A (ES9) | mouse IgM | 81 | 54 | 27 | 20 |
| Anti-B (LB2) | mouse IgM | 108 | 72 | 36 | 27 |
| Anti-A,(B) ES15 | mouse IgM | 127 | 85 | 42 | 32 |
| Anti-D (LDM3) | human IgM | 34.8 | 17.4 | 8.7 | 4.35 |
| Anti- D (ESD1) | human IgG | 300 | 200 | 100 | 75 |
| Con A | lectin | 1000 | 500 | 250 | 125 |
| PBS | neg. control | n/a | n/a | n/a | n/a |

### 4.2.1.3 ABO Typing Microplate Assay

In order to assess the solid-phase suitability of the available ABO blood typing antibody probes, it was decided to use an ELISA type format. A polystyrene microplate has a highly hydrophobic surface and will therefore bind hydrophobic molecules, such as antibodies. Polystyrene microplates were used to immobilise various blood group antibodies using the Blood Typing ELISA method. Each probe (**Table 4.1,** 'Dil 1' only) was immobilised in triplicate. Group B cells were added and incubated in appropriate wells, followed by FITC ConA in PBS-Mn/Ca.

**Table 4.2.** Identity of plates used in experiment detailed in 4.2.1.3, with description of cells added and labelling method.

| Target description | Plate identity |
|---|---|
| Group A cells prelabelled with FITC ConA (indirect primary labelling) | Plate 1 |
| Group B cells prelabelled with FITC ConA (indirect primary labelling) | Plate 2 |
| Group A cells added, followed by FITC ConA (indirect secondary labelling) | Plate 3 |
| Group B cells added, followed by FITC ConA (indirect secondary labelling) | Plate 4 |
| PBS only used followed by FITC ConA (to determine NSB) | Plate 5 |
| FITC anti-mouse IgM added (to determine if probes are present) | Plate 6 |

No specific binding of erythrocytes was apparent by either fluorescence or microscopic examination. One possible reason for negative results was that antibody probes were not immobilised on the microplate. Therefore, the following experiment was performed to investigate whether antibodies were attached to the plate using this method. The same probe antibodies as before were used (**Table 4.1**), but the target solutions were as described in **Table 4.2,** which also details the plate identities for analysis purposes.

In plates 1 and 2, clumps of cells could be seen macroscopically. Plates 3 and 4 were examined prior to the addition of the secondary labelling solution. In plate 3, to which group A cells had been added, wells containing Anti-A LA2, Anti-A ES9 and Anti-A,(B) ES15 contained bound erythrocytes when examined macroscopically. In plate 4 containing group B cells, wells containing Anti-B (LB2) and Anti-A,(B) also contained bound cells. When plates 3 & 4 were re-examined after incubation with FITC ConA, no cells were evident even microscopically. When examined in a fluorescence reader, the plates gave no distinct readings for wells in plates 1 to 5. In plate 6, where FITC anti-mouse IgM was added, low-level reactions were found (data not shown). As specific binding of erythrocytes to the plate was seen, it was concluded that antibody was being immobilised to the microplates.

In summary, this experiment established; that antibody had been coated onto the microplates, erythrocytes can bind to antibodies immobilised to a solid support, and that indirect secondary labelling somehow interferes with binding to antibody. Due to problems with labeling methods, another suitable method of detection was critical to the development of a blood typing microarray.

## 4.3 Direct Detection Using FITC Labelled Erythrocytes

### 4.3.1 Evaluation of Directly FITC Labelled Erythrocytes

A method to directly FITC label erythrocyte membranes, developed by Dr. D. Pepper, was adopted. Several methods were used to assess the FITC labelling of the erythrocytes. Cells were analysed by FACS analysis to determine the labelling efficiency. The results detailed in **Table 4.3** show that, regardless of blood group, the FITC labelling is over 99 % efficient.

**Table 4.3.** FACS analysis of directly FITC labelled erythrocytes.

| Label & quantity | % of total labelled |
|---|---|
| Unlabelled group A cells | 0.02 |
| FITC labelled group A cells | 99.92 |
| FITC labelled group B cells | 99.95 |
| FITC labelled group O cells | 99.92 |

The cells described in the above table were also smeared onto glass slides, dried and then scanned. High levels of fluorescence were seen from labelled cells, and a low level of autofluorescence was evident with unlabelled cells.

As the previous experiment had shown that erythrocytes had bound specifically in a blood typing microplate assay, a microplate blood typing assay was performed using FITC labelled erythrocytes. This would determine if the labelled cells could bind to immobilised antibody and if the FITC labelling method could be used for detection. While erythrocyte binding was visible, no fluorescent signals were found. It was concluded that the plate reader was not optimal for FITC detection in this format.

The microplate assay also demonstrated that, unlike the FITC ConA, direct FITC labelling does not block binding of cells to immobilised antibody, as cells could be seen bound to the plate both macro- and microscopically. It was decided to use the direct FITC labelling of erythrocytes in a blood typing microarray, as the equipment available was specifically designed to detect and read fluorescent signals from microarray slides.

### 4.3.2 Blood Typing Microarray Using Directly FITC Labelled Erythrocytes

Prior to this stage, blood typing microarrays had been unsuccessful. As directly labelled erythrocytes had bound to the blood typing microplate, it was decided to use the cells in a blood typing microarray. Due to a lack of erythrocyte binding in previous microarray experiments, it was felt that allowing the cells to be mixed over the microarray surface in solution might increase the opportunity for interaction and binding.

**Table 4.4.** Probes spotted for blood typing microarray using different reaction volumes, concentration in $\mu$g/ml.

| Protein/ Antibody ID | Protein /Ab type | Conc. DIL1 | Conc. DIL2 | Conc. DIL3 | Conc. DIL4 |
|---|---|---|---|---|---|
| Anti-A (LA2) | mouse IgM | 55 | 37 | 18 | 14 |
| Anti-A (ES9) | mouse IgM | 81 | 54 | 27 | 20 |
| Anti-B (LB2) | mouse IgM | 108 | 72 | 36 | 27 |
| Anti-A,(B) ES15 | mouse IgM | 127 | 85 | 42 | 32 |
| Anti-D (LDM3) | human IgM | 34.8 | 17.4 | 8.7 | 4.35 |
| Anti-D (ESD1) | human IgG | 300 | 200 | 100 | 75 |

In all previous microarray experiments detailed in this thesis, a 25 $\mu$l volume of target solution has been used. This was added under cover slip on which the edges

are 'lifted' (i.e. heightened) allow a volume to be pulled under. These are called Lifter Slips™ (Erie Scientific, U.S.A.) and they allow limited mixing of the solution on the solid surface. The use of a larger target solution volume may increase both the movement and allow greater mixing, and therefore more efficient binding. For this purpose Hybridisation Chambers from Schleicher & Schuell (Germany) were used which can hold a 500 $\mu$l volume. To allow mixing in this experiment, a 450 $\mu$l volume was used, and the slides were rotated throughout the incubation period. The use of both reaction volumes was compared to determine if a reaction between cells and specific antibody is facilitated by this method. Antibody probes printed for this experiment are detailed in **Table 4.4**.

The target erythrocyte solutions were FITC labelled group $A_1$, B and O erythrocytes, and unlabelled group $A_1$ was also used as a control, and all were RhD negative. The values from LDM3 and ESD1 spots (anti-D antibodies) were used to calculate noise for the S/N ratio. The results are presented in **Figures 4.2a-c.**

Group $A_1$ and group B cells have bound specifically to the microarrays when using both the 25 $\mu$l and 450 $\mu$l volumes. However, **Figures 4.2a** and **4.2b** clearly demonstrate that the larger volume and mixing substantially increase the S/N values both from group $A_1$ and group B cells, over that obtained using the 25 $\mu$l volume. **Figure 4.2a** also demonstrates the level of autofluorescence present when unlabelled cells are used, and is presented against the S/N values achieved using the FITC labelling. Here, specific reactions are also increased when using a larger volume and mixing. **Figure 4.2c** demonstrates the reactions using two group O labelled cells

(volume 450 $\mu$l) where no binding is expected, and consequently reactions are low. The results demonstrate the first successful blood typing microarray performed in this work, and it was apparent that blood typing could be successfully performed using microarrays. This was a defining moment, proving the principle and potential for blood typing microarrays.

The larger reaction volume, along with mixing, had allowed increased Ab-Ag interactions and subsequently increased the S/N values through enhanced binding to the array.

Although the smaller 25 $\mu$l volume had also allowed specific interaction to take place, the Hybridisation Chambers were used in subsequent experiments where appropriate (Slide Processing Method D) as these provided better results.

As the data shows, the scan results gave detectable fluorescence values, which could be used to give S/N ratios demonstrating specific blood typing reactions. As the spot sizes were one mm in diameter, the binding of the cells could be checked visually. When examined microscopically differential cell binding to various spots could be seen and to demonstrate this, photographs were taken of the microscopic images.

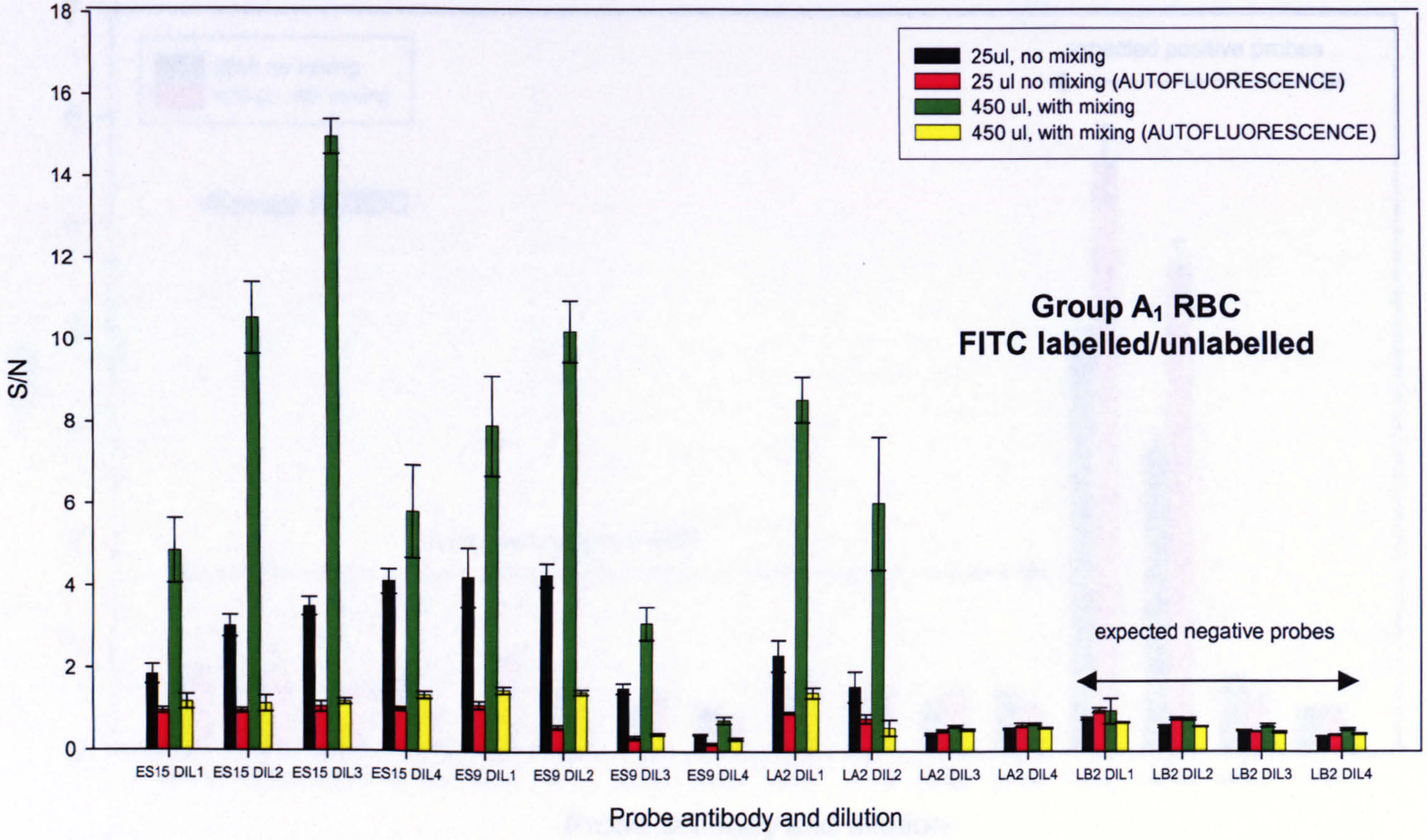



**Figure 4.2a**. Reactions of directly FITC labelled and unlabelled group $A_1$ erythrocytes with various probe antibodies, comparing reactions using 25 $\mu$l volume with no mixing (Lifter Slips™), and a 450 $\mu$l volume with mixing (Hybridisation chambers). Red and yellow bars represent autofluorescence from erythrocytes. Slide type poly-L-lysine, slide reps 1, pins 700 $\mu$m, probes Table 4.4, probe reps 3, SPM C/D; blocker PBS-milk, target/volume: FITC group $A_1$/unlabelled group $A_1$ RBC/25 $\mu$l/450$\mu$l, incubation 120 min, static/mixing, scanning method B.

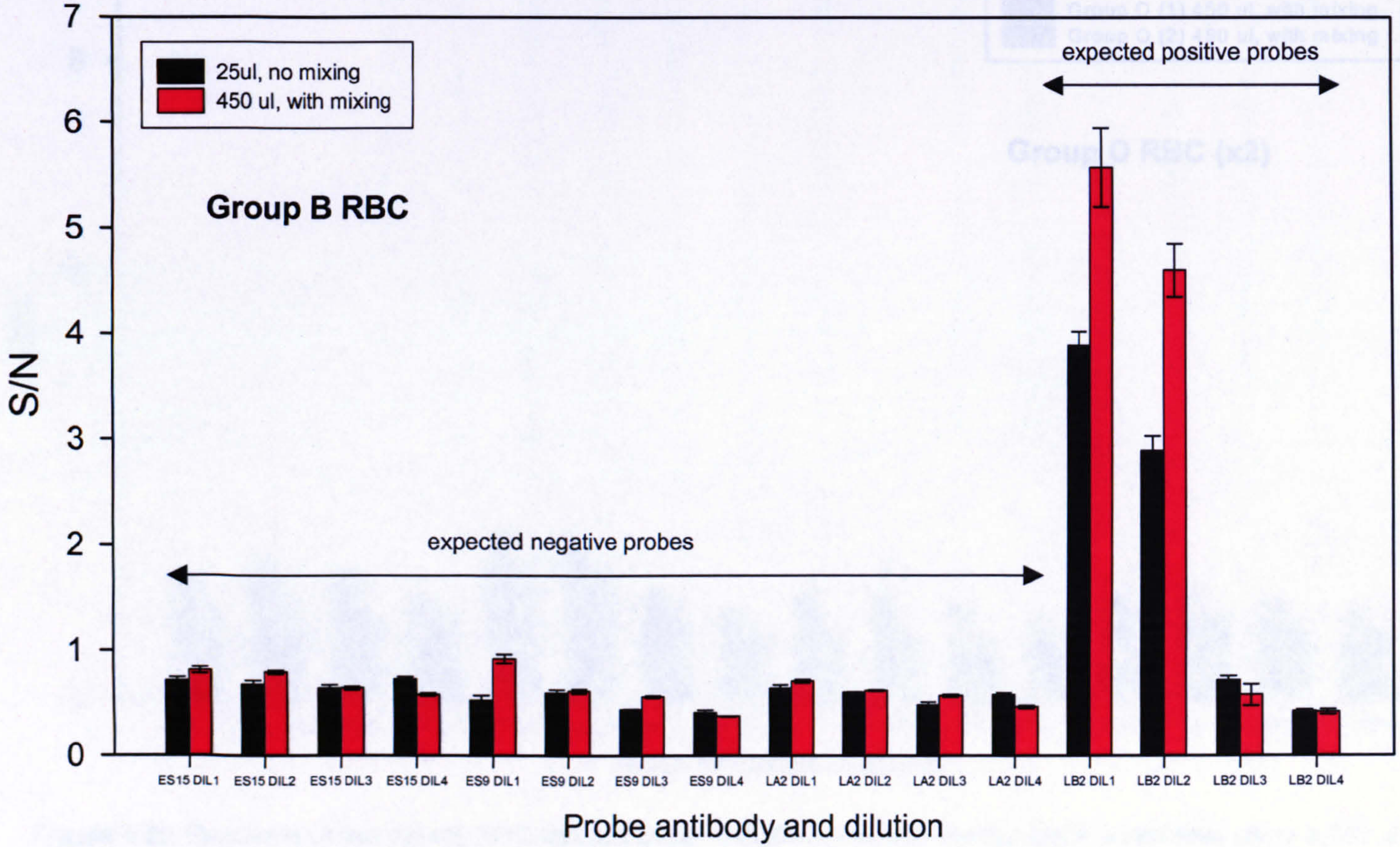



**Figure 4.2b**. Reactions of directly FITC labelled group B erythrocytes with various probe antibodies, comparing reactions using 25 $\mu l$ volume with no mixing (Lifter Slips™), and a 450 $\mu l$ volume with mixing (Hybridisation chambers). Slide type poly-L-lysine, slide reps 1, pins 700 $\mu$m, probes Table 4.4, probe reps 3, SPM C/D; blocker PBS-milk, target/volume: FITC group B RBC/25 $\mu l$/450$\mu l$, incubation 120 min, static/mixing, scanning method B.

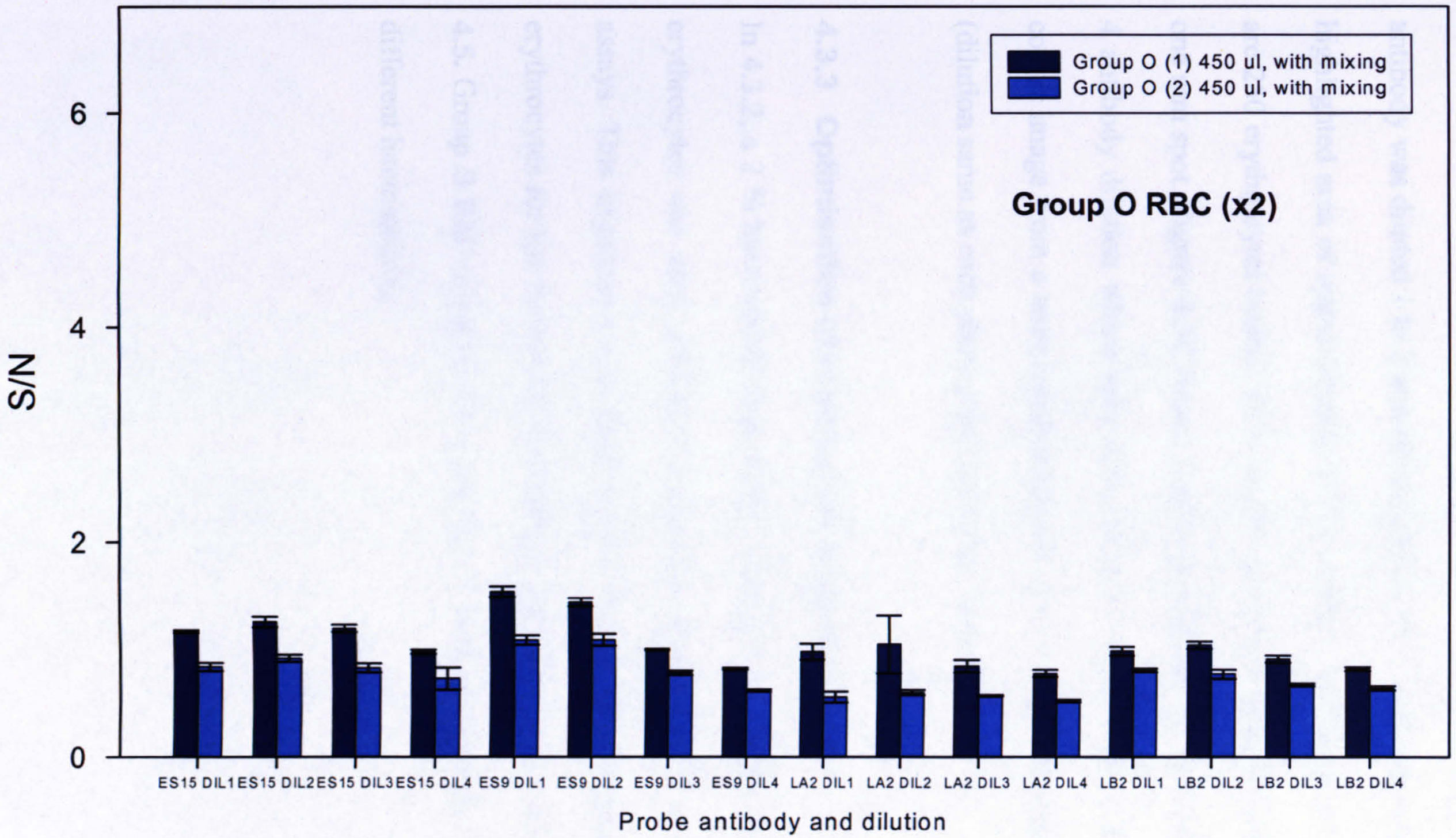



**Figure 4.2c.** Reactions of two directly FITC labelled group O erythrocytes with various probe antibodies, using a 450 $\mu$l volume with mixing (Hybridisation chambers). Slide type poly-L-lysine, slide reps 1, pins 700 $\mu$m, probes Table 4.4, probe reps 3, SPM D; blocker PBS-milk, target/volume: FITC group O RBC/450$\mu$l, incubation 120 min, mixing, scanning method B.

In **Figure 4.3, A** shows a spot where the capture of erythrocytes is almost saturated, the antibody is spotted neat. By calculation of area, it is estimated that a one mm spot (area of 0.785 $mm^2$) could theoretically bind 15,700 cells (if approximate area of cell is $5 \times 10^{-5}$ $mm^2$, and all lay 'flat' on the surface). **Figure 4.3B** shows a spot where the antibody was diluted 1 in 2 and subsequently fewer cells are bound. This figure has a highlighted area of approximately 1/16th of the whole spot, in which it is estimated are 250 erythrocytes bound. This would equate to a total of 4,000 cells bound to the one mm spot. **Figure 4.3C** shows a more dilute antibody spot and **Figure 4.3D** a 1 in 4 antibody dilution where very few cells are bound. **Figure 4.3E** shows a pseudo colour image from a scan representing the scan image from the photographed spots (dilution same as each photograph above the spots).

### 4.3.3 Optimisation of Erythrocyte Concentration

In 4.3.2, a 2 % haematocrit suspension (estimated to contain $2 \times 10^5$ cells per $\mu$l) of erythrocytes was used, adopted from a concentration used in routine blood typing assays. This experiment was performed to determine the optimal concentration of erythrocytes for the further development of the microarray method detailed in **Table 4.5.** Group B RhD negative cell suspensions were prepared in blocking buffer at the different haematocrits.

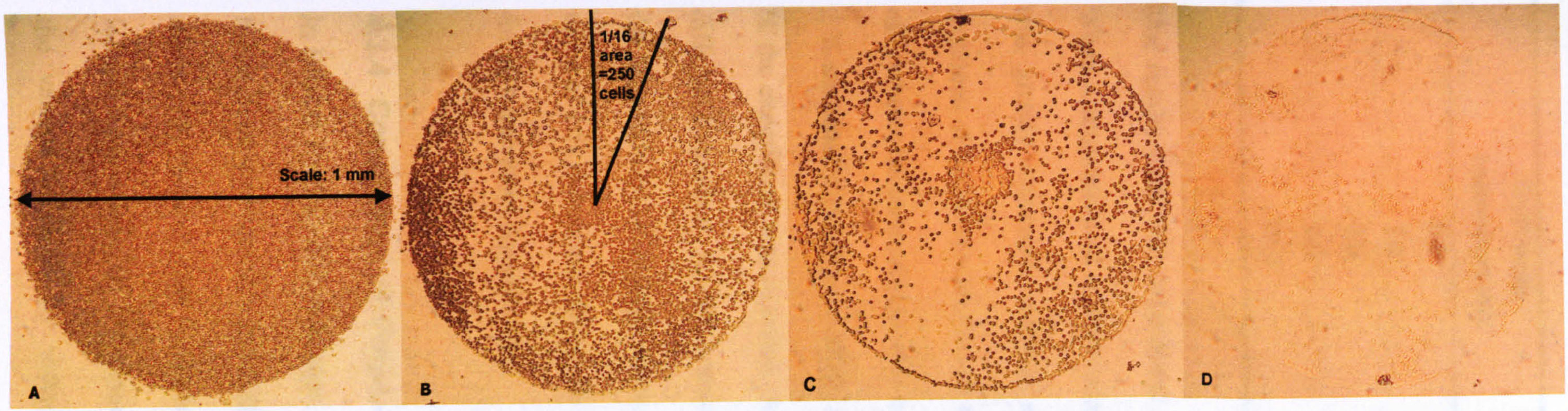



**Figure 4.3.** Photographs of microarray spots with Anti-A as probe antibody and Group $A_1$ erythrocytes bound (A-D), and a representative pseudo colour scan image (not actual scan from spots) (E). Scale is estimated.

**Table 4.5.** Haematocrit and estimated number of cells present in reaction volume of 450 $\mu l$.

| Erythrocyte haematocrit (%) | Number of cells in 450 $\mu l$ volume (estimated) |
|---|---|
| 0.5 | $2.25 \times 10^7$ |
| 1.0 | $4.50 \times 10^7$ |
| 2.0 | $9.00 \times 10^7$ |
| 5.0 | $2.25 \times 10^8$ |
| 10.0 | $4.50 \times 10^8$ |

The results from this experiment are presented in **Figure 4.4a** and **Figure 4.4b.** When a suspension of erythrocytes containing 0.5 or 1.0 % cells was used, the S/N was greatly increased, without an increase in NSB, demonstrated by minimal reactions of the cells with Anti-A and Anti-A(B) on the array. Lower concentrations of erythrocytes increases the S/N value by up to 900 % on reactions compared with a 2 % suspension (in **Figure 4.4a**). As there was such a variance between 1.0 and 2.0 % the experiment was repeated. Again, group B erythrocytes were used, but from a different individual. As demonstrated in **Figure 4.4b**, a 1 % suspension again gives the highest level of binding, where 1 % S/N is 130 and 2 % is 102. The haematocrit value of 1 % had repeatedly given the highest S/N values and was, therefore, selected for all subsequent experiments.

### 4.3.4 Comparison of Poly-L-lysine and Polyacrylamide Slides For Use in Blood Typing Microarrays

It has been demonstrated in Chapter 3 that both poly-L-lysine and polyacrylamide slides gave good results in model microarray antibody-antigen experiments. This looked at the interaction of various probes and ligands, and the level of interaction on the various surfaces, by using anti-species antibodies. Polyacrylamide slides

demonstrated a superior S/N ratio to others tested. The polyacrylamide is a gel matrix that supplies a three dimensional scaffold to help maintain structural integrity, holding the large antibody molecules in their quaternary structure and consequently is likely to retain proteins after washing and blocking procedures. In contrast, poly-L-lysine slides bind proteins by electrostatic forces on a two-dimensional surface but were also shown to give suitable results

Here, the two slide surfaces were evaluated for their use in blood typing, antibody-cell microarrays. It was expected that the cells would be too large to penetrate the polyacrylamide matrix to interact with probe antibodies, but it was important to evaluate this given the superior performance of the slides in Chapter 3.

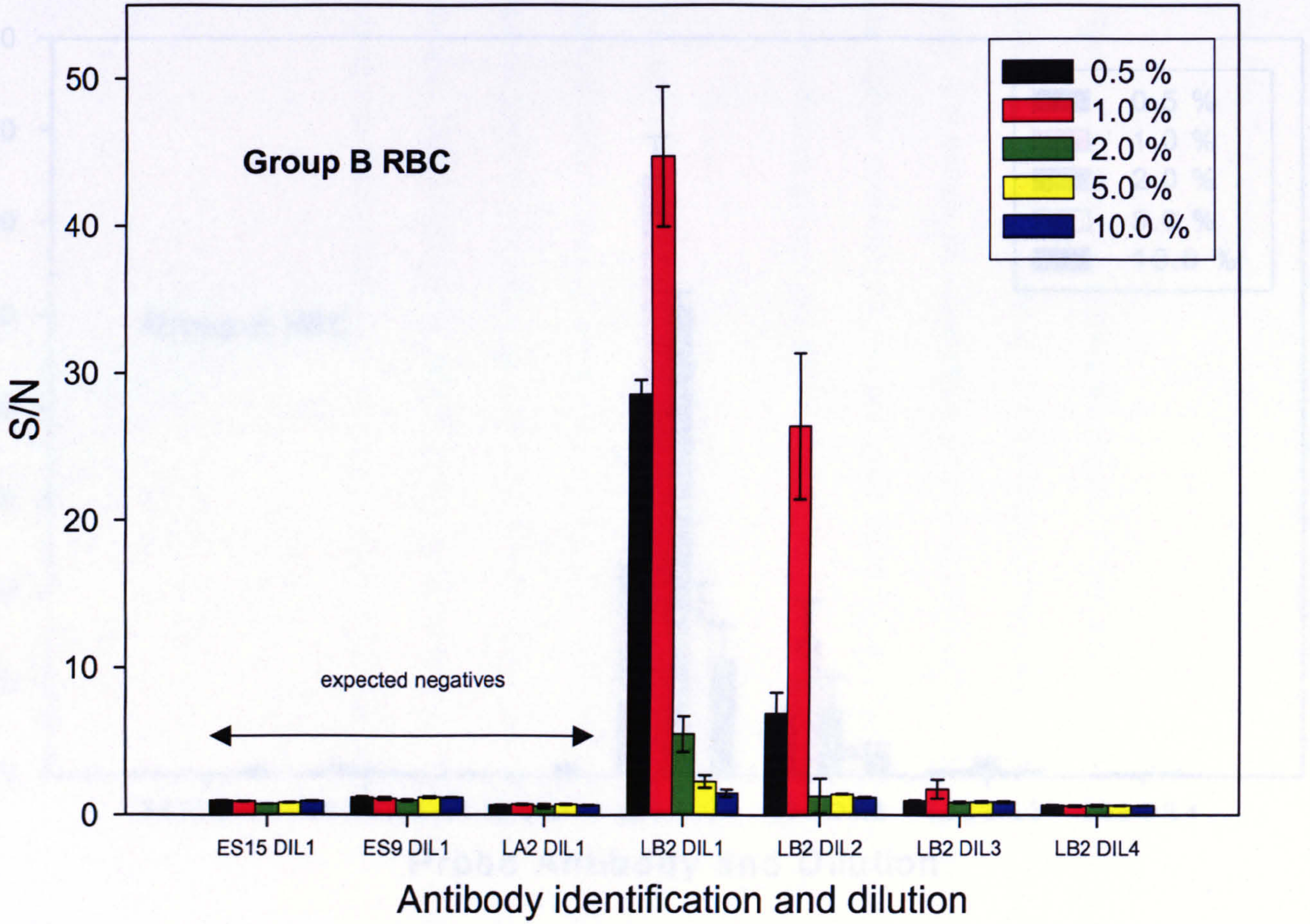



**Figure 4.4a**. Comparison of erythrocyte haematocrit effect on binding to blood typing microarray, group B erythrocytes used
Slide type poly-L-lysine, slide reps 1, pins 700 $\mu$m, probes Table 4.4, probe reps 3, SPM D; blocker PBS-milk, target/volume: FITC group B RBC/450$\mu$l, incubation 120 min, mixing, scanning method B.

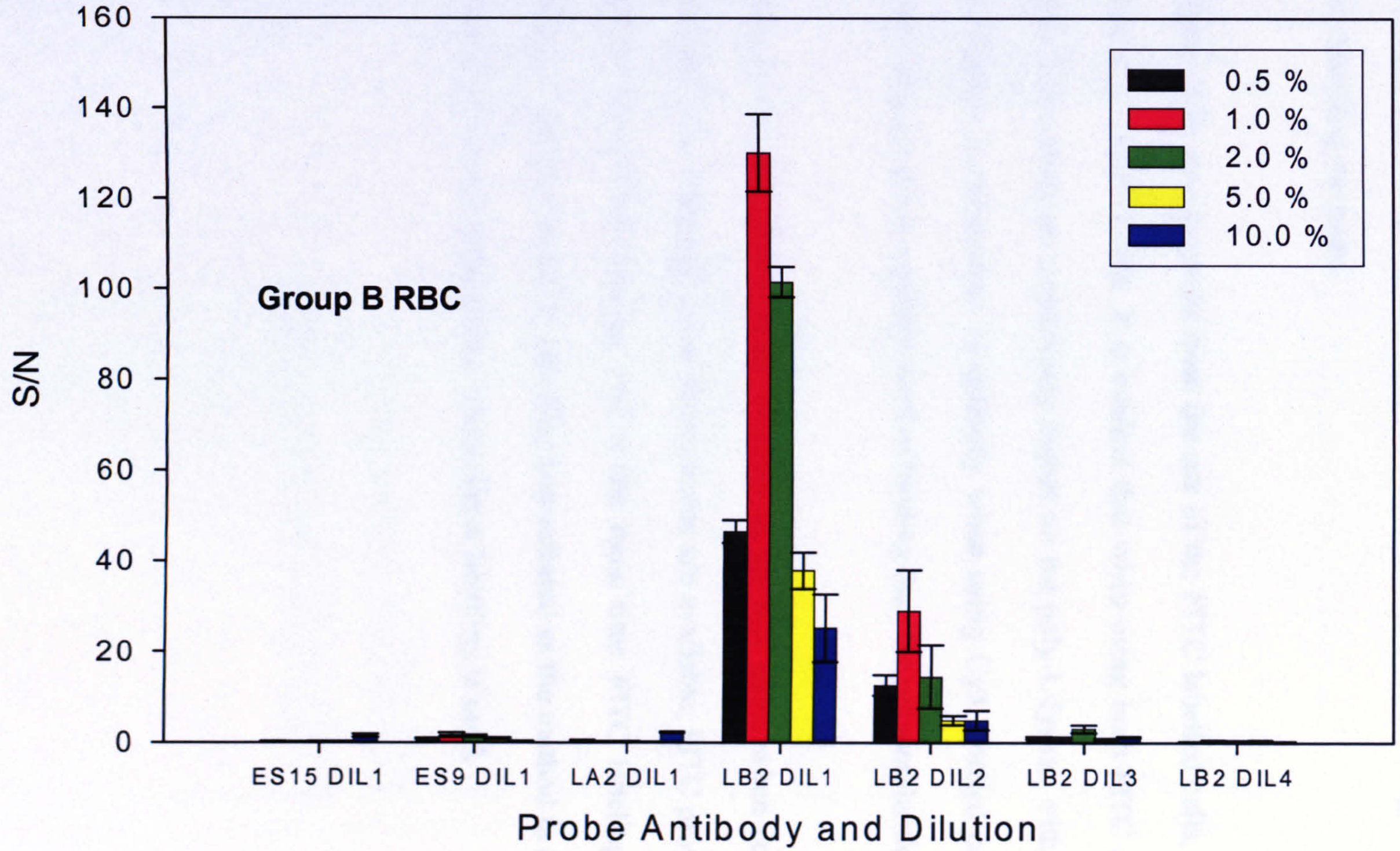



**Figure 4.4b**. Repeat of comparison of erythrocyte haematocrit effect on binding to blood typing microarray, group B erythrocytes used.
Slide type poly-L-lysine, slide reps 1, pins 700 $\mu$m, probes Table 4.4, probe reps 3, SPM D; blocker PBS-milk, target/volume: FITC group B RBC/450$\mu$l, incubation 120 min, mixing, scanning method B.

Antibodies (described in **Table 4.4**) were printed onto poly-L-lysine and polyacrylamide slides (Hydrogel slides, Packard BioScience). FITC labelled erythrocytes were used, and also Cy3 labelled erythrocytes, which were prepared by Dr. D. Pepper. Labelled erythrocytes were prepared from the same sample of group $A_1$ cells, and used at the same concentration (1 %), thus allowing a comparison of the two labelling methods.

**Figure 4.5a** shows results from the use of the FITC labelled cells, and **Figure 4.5b** from Cy3 labelled cells. It is evident that when using both FITC and Cy3 labelled cells, S/N values are consistently higher on the poly-L-lysine, with the exception of the highest concentration of antibody when using Cy3 labelled cells. The poly-L-lysine surface gives a greater level of binding than the polyacrylamide matrix.

The use of Cy3 gives a small increase in the S/N ratios when compared to FITC labelled cells. Although other fluorophores are available, FITC provides satisfactory results from direct labeling, and at the same time FITC labeling is substantially cheaper. Due to this, FITC labelling was selected as the method in subsequent blood typing microarray experiments where direct labelling is used.

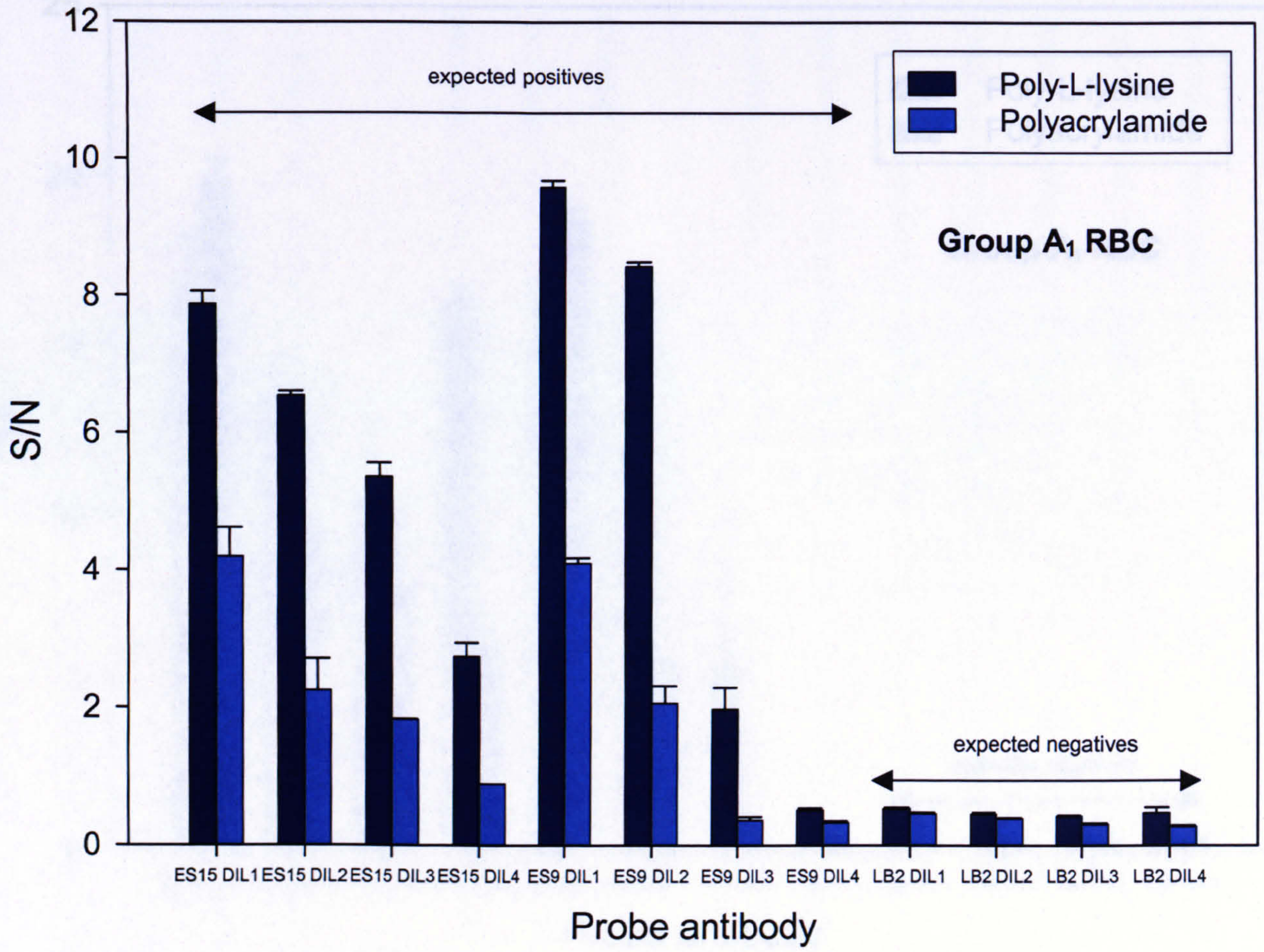



**Figure 4.5a.** Comparison of blood typing reactions performed on poly-L-lysine and polyacrylamide slides, using FITC labelled group $A_1$ erythrocytes. Slide type poly-L-lysine/polyacrylamide, slide reps 1, pins 700 $\mu$m, probes Table 4.4, probe reps 4/2, SPM D; blocker PBS-milk, target/volume: FITC group $A_1$ RBC/450$\mu$l, incubation 120 min, mixing, scanning method B.

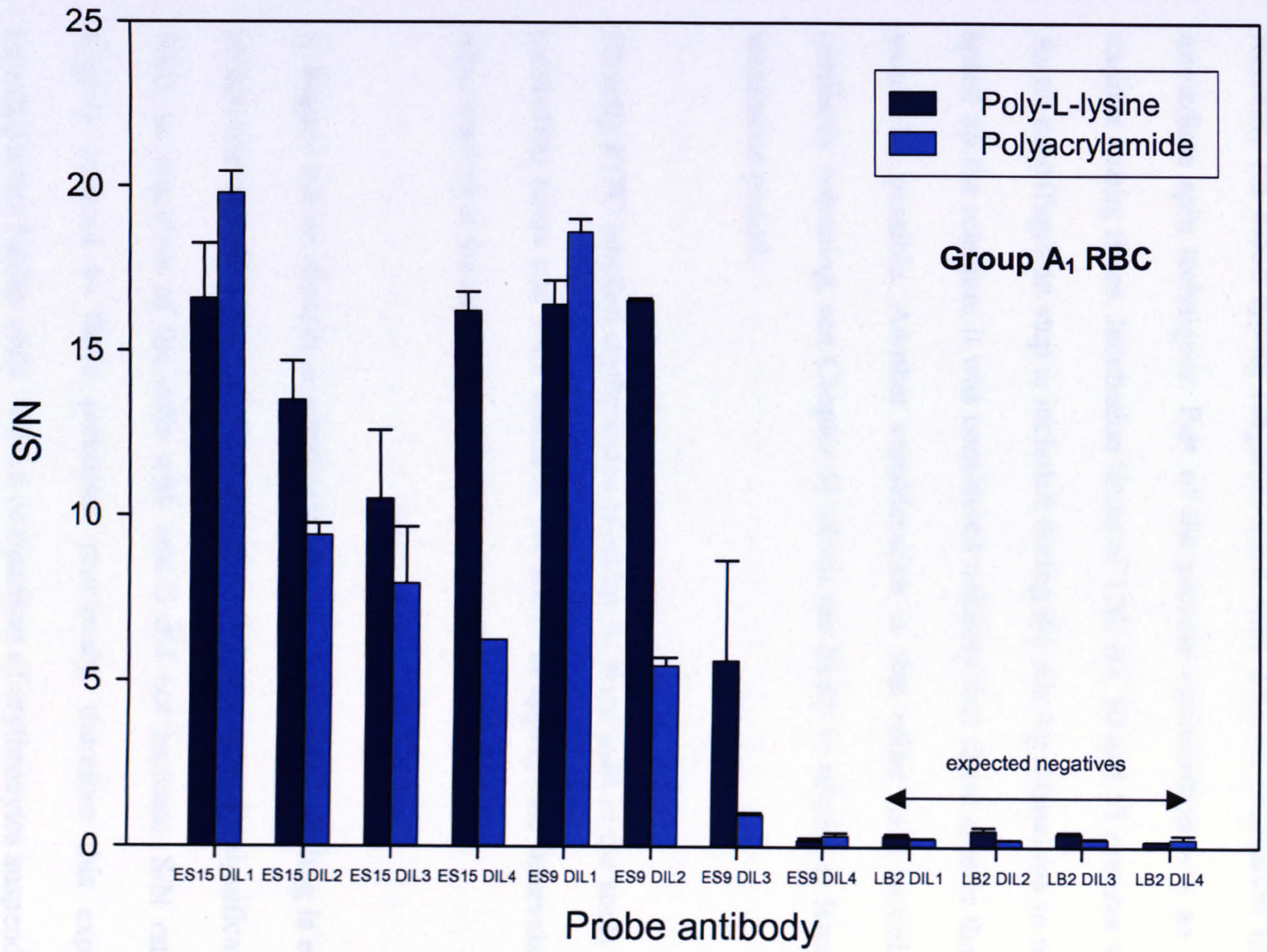



**Figure 4.5b.** Comparison of blood typing reactions performed on poly-L-lysine and polyacrylamide slides, using Cy3 labelled group $A_1$ erythrocytes. Slide type poly-L-lysine/polyacrylamide, slide reps 1, pins 700 $\mu$m, probes Table 4.4, probe reps 4/2, SPM D; blocker PBS-milk, target/volume: Cy3 group $A_1$ RBC/450$\mu$l, incubation 120 min, mixing, scanning method B.

### 4.3.5 Investigation of Optimal Incubation Time for Blood Typing Microarrays

In Ab-Ag interactions, time of incubation can greatly influence the specificity and sensitivity of the test. So far in this work, a two-hour incubation time has been used when performing blood typing microarrays. The antibodies used in this work are routinely (in blood typing reagents) used with reduced incubation times, some in immediate spin techniques. Part of the process optimisation was an evaluation of various testing times. Incubation times of 120, 60, 30 and 15 minutes were selected. As no centrifugation step is included during the Ab-Ag interaction in microarrays to speed up the reaction, it was considered unlikely that times shorter than 15 minutes would be possible. Another consideration is that other tests would be included (antibody screening, see Chapter 6) which are likely to require at least a 15-minute incubation period.

Directly FITC labelled erythrocytes of group $A_1$ were used in the study of a range of incubation times and were added to the slides at appropriate intervals so all slides were washed at the same time.

In **Figure 4.6** no distinct or consistent pattern of improved binding is evident at any incubation time. However, no time period used demonstrated a significant increase in NSB, as reactions of the cells with anti-B did not increase. S/N ratios appeared slightly reduced to those presented previously, therefore this experiment was repeated using further cells. Also, a comparison of erythrocytes suspended in normal ionic strength saline (NIS) and low ionic strength saline (LIS) was included. The

results of this experiment are **Figure 4.7a** and **Figure 4.7b.** The results from 120-minute incubations are not included.

In **Figure 4.7a** an incubation time of 60 minutes is clearly optimal, giving S/N ratios of over 10. In **Figure 4.7b** it appears that there is less differentiation between incubation times, with the 15 minute incubation time performing as well as 60 minutes.

Using the same batch of slides, this experiment was repeated using another batch of FITC labelled group $A_1$ cells, performed at all four incubation times using NIS and LIS (data not shown). The NIS results showed that a reduction from 120 minutes to either 60 or 30 minutes gives an increased level of binding. An incubation time of 60 minutes was selected as a suitable incubation time if using cells suspended in PBS. In blood grouping serological techniques, it is common that a time period of 45-60 minutes is optimal when using normal ionic strength saline.

When using low ionic strength saline, a time period of 15-20 minutes is usually recommended. Using LIS at 15 minutes in **Figure 4.7b**, the 15-minute incubation looks as though it may be optimal, but this was not confirmed by subsequent results (not shown).

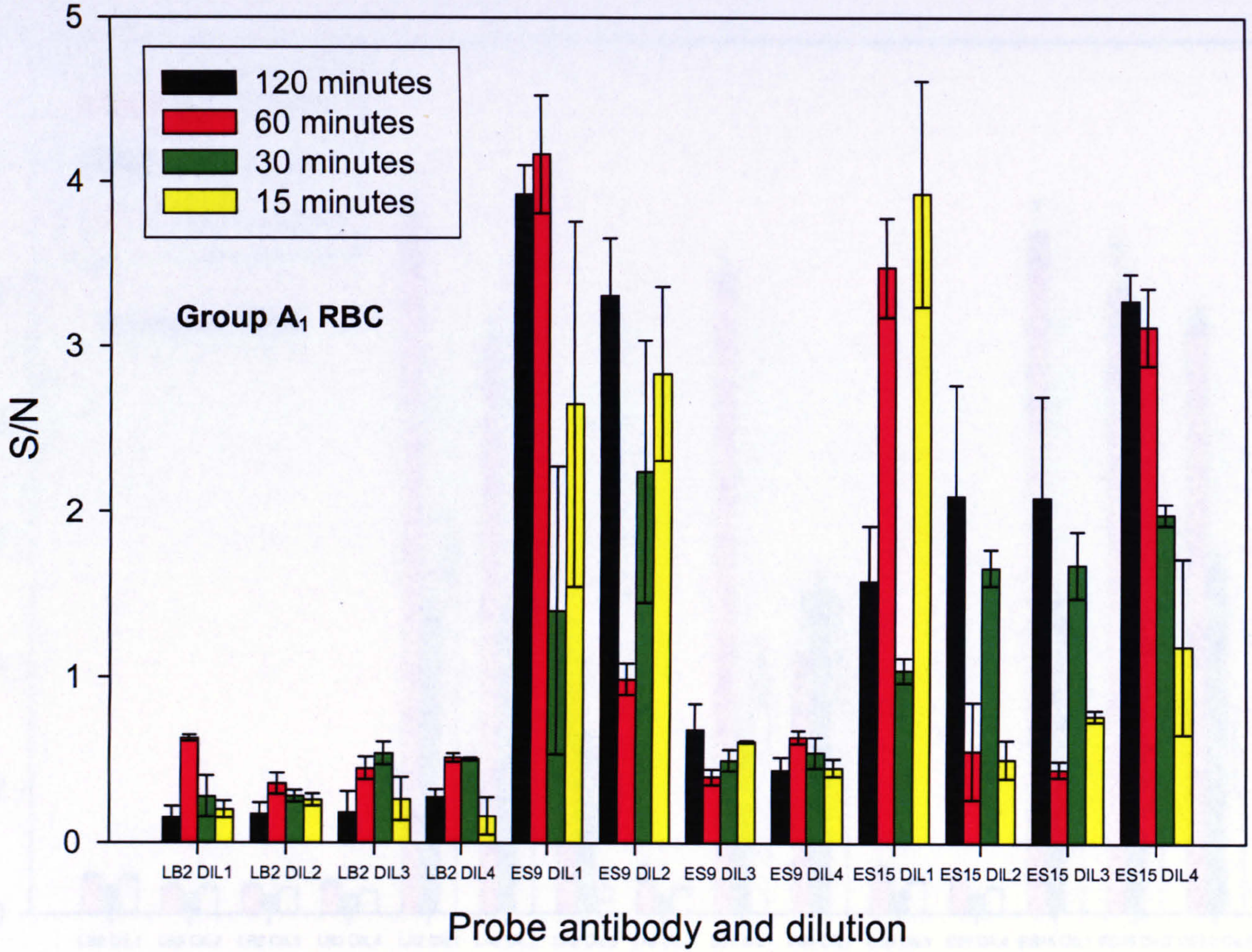



**Figure 4.6.** Comparison of varying incubation times, using group $A_1$ erythrocytes.
Slide type poly-L-lysine, slide reps 1, pins 700 $\mu$m, probes Table 4.4, probe reps 3, SPM D; blocker PBS-milk, target/volume: FITC group $A_1$ RBC/450$\mu$l, incubation time varied, mixing, scanning method B.

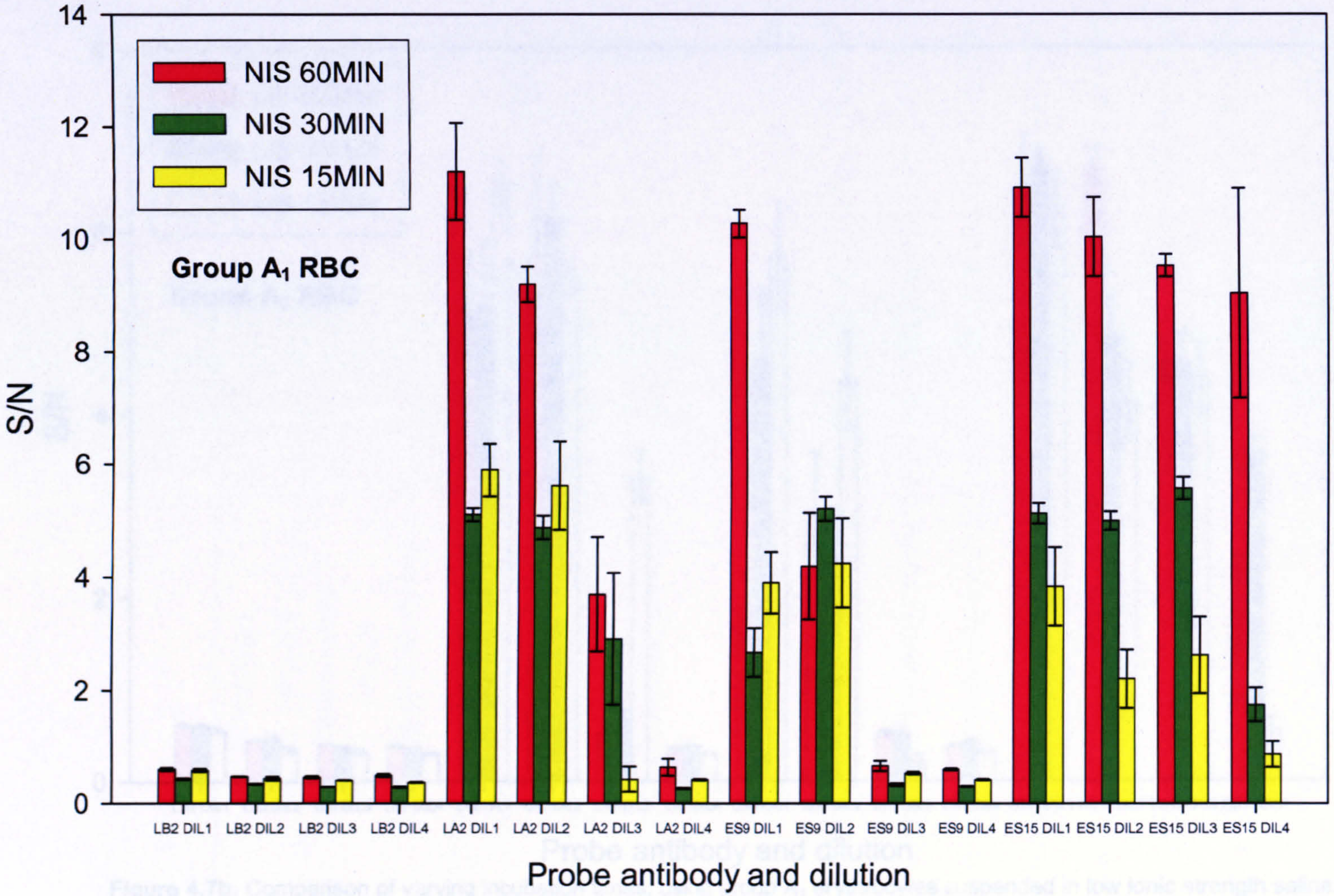



**Figure 4.7a.** Comparison of varying incubation times, using group $A_1$ erythrocytes suspended in normal ionic strength saline. Slide type poly-L-lysine, slide reps 1, pins 700 $\mu$m, probes Table 4.4, probe reps 3, SPM D; blocker PBS-milk, target/volume: FITC group $A_1$ RBC/450$\mu$l, incubation time varied, mixing, scanning method B.

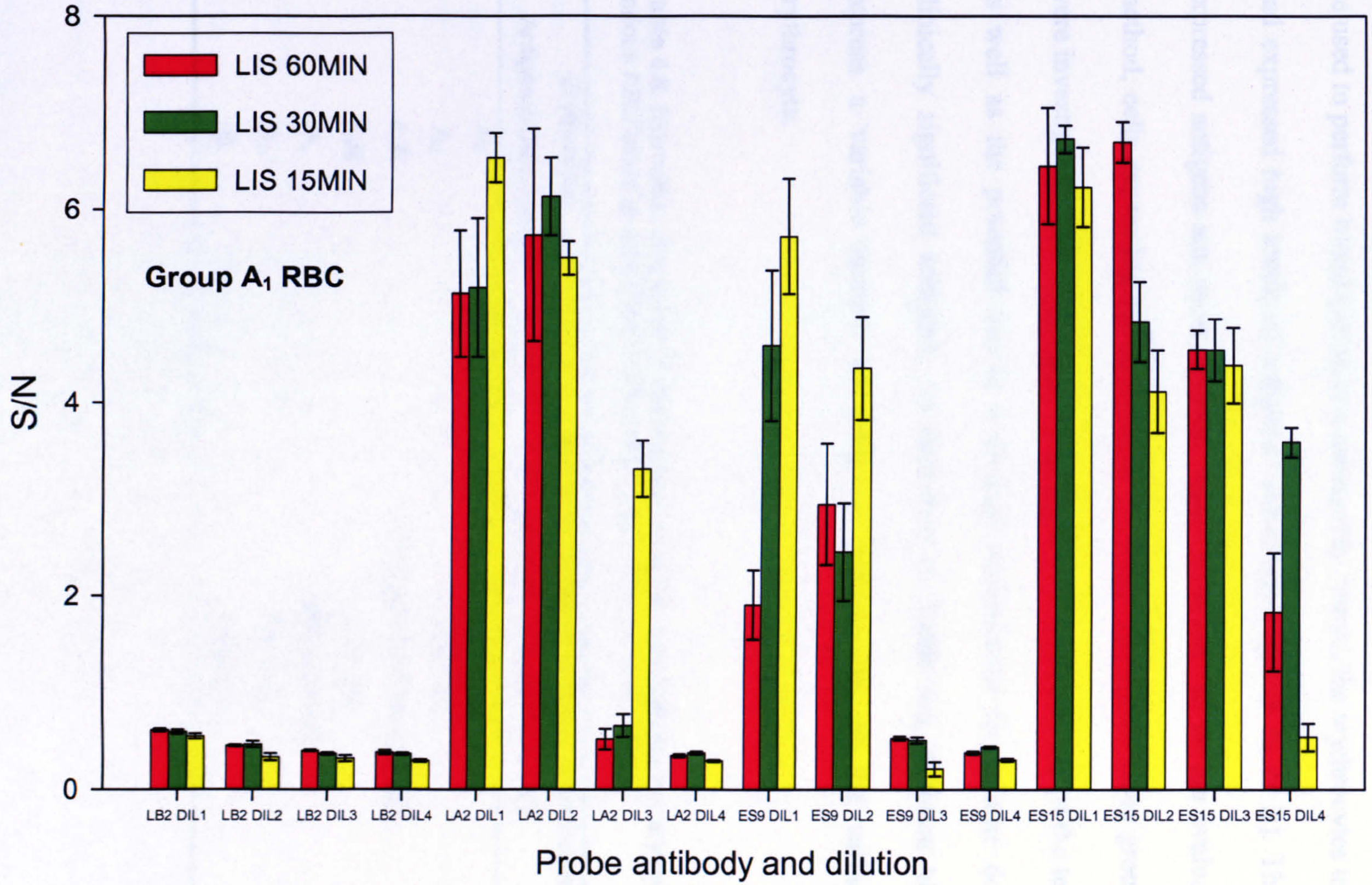



**Figure 4.7b.** Comparison of varying incubation times, using group $A_1$ erythrocytes suspended in low ionic strength saline. Slide type poly-L-lysine, slide reps 1, pins 700 $\mu$m, probes Table 4.4, probe reps 3, SPM D; blocker PBS-milk, target/volume: FITC group $A_1$ RBC/450$\mu$l, incubation time varied, mixing, scanning method B.

### 4.3.6 Interaction of Probe and Erythrocytes Expressing Reduced Levels of Antigen

While previous experiments have shown that the developed microarray method can be used to perform blood typing in a microarray format, the erythrocytes tested so far had expressed high levels of antigenic determinant (group $A_1$ or B). These highly expressed antigens are, most probably, easier to detect. To further evaluate the test method, cells expressing lower levels of clinically significant blood group antigens were investigated. This would give an indication of the sensitivity of the test method, as well as the potential use in a clinical environment for routine detection of clinically significant antigens. As described in **Table 4.6**, different blood types express a variable number of antigenic determinants on the surface of the erythrocyte.

**Table 4.6.** Estimation of number of blood group antigenic determinants per erythrocyte of various ABO blood groups (Econimidou *et al*, 1967; Cartron *et al*., 1974).

| Erythrocyte Antigenic Determinant | Average number of antigenic determinant sites per erythrocyte (x $10^6$) |
|---|---|
| $A_1$ | 0.81-1.17 |
| $A_2$ | 0.24-0.29 |
| $A_1B$ | 0.46-0.85 (A), 0.31-0.56 (B) |
| $A_2B$ | 0.12 (A) |
| $A_x$ | 0.0014–0.0103 |
| $A_3$ | 0.007–0.1 |
| B | 0.62-0.83 |

As discussed in Chapter 1 (1.3.1), it is mandatory to detect certain blood group antigens; therefore a new testing system must be sensitive enough to detect low levels of antigen. Erythrocyte types selected for analysis were B, $A_2$, $A_1B$ and $A_x$. The data from the experiment is in **Figure 4.8**. All probe antibodies were spotted at different concentrations. Consequently, the data here is presented in two alternative ways: the complete data in one graph (**Figure 4.8**), and then each cell against each antibody at one concentration (**Figure 4.9**). The concentration selected for presentation is where the antibody probes are at the concentration detailed in yellow **Table 4.7**. These were selected as most comparable concentrations.

**Table 4.7.** Probes selected for presentation in data in Figures 4.13 and 4.14. Those selected at most similar $\mu$g/ml are highlighted in yellow. Anti-D were used to calculate noise.

| Probe/ antibody ID | Protein/ Ab type | Conc. DIL1 | Conc. DIL2 | Conc. DIL3 | Conc. DIL4 |
|---|---|---|---|---|---|
| Anti-A (LA2) | mouse IgM | 55 | 37 | 18 | 14 |
| Anti-A (ES9) | mouse IgM | 81 | 40.5 | 20.25 | 10.13 |
| Anti-B (LB2) | mouse IgM | 108 | 54 | 36 | 27 |
| Anti-A,(B) ES15 | mouse IgM | 127 | 63.5 | 31.75 | 15.9 |
| Anti-D (LDM3) | human IgM | 34.8 | 17.4 | 8.7 | 4.35 |
| Anti- D (ESD1) | human IgG | 300 | 200 | 100 | 75 |

**Figure 4.9a** demonstrates specific binding of group B erythrocytes to anti-B LB2 with a S/N ratio of 12. **Figure 4.9b** gives the result from the $A_1B$ cells. $A_1B$ erythrocytes, on average, have fewer A and B antigen sites than a normal group $A_1$ or group B (see **Table 4.6**). However, the antigen density is still high enough to give strong binding (S/N values of 15 to 20) to the microarray. Differences in either the antibody concentration or affinity to antigen can be seen in this data.

**Figure 4.9c** shows the results from the group $A_2$ cell. Although specific reactions are present with both anti-A on the microarray, the S/N ratios are low at around 2. The cells give only <1 S/N with the anti-A(B) ES15. This antibody reacts more weakly with cells of the $A_2$ subgroup (Moore *et al.*, 1984). Cells of type $A_x$ have comparatively low numbers of antigen sites on the cell surface (see **Table 4.6**). **Figure 4.9d** shows that the only result above a S/N ratio of 1 is that with anti-A(B) ES15, which is described by Moore *et al.* (1984) as an antibody that reacts well with $A_x$ cells. This cell line is used in many commercially available products as it is known to be excellent for the detection of $A_x$ cells. It is usually blended with anti-A and anti-B cell lines to give a product that detects $A_1$ and B cells equally. In **Figure 4.8,** the reaction with $A_x$ cells gives a higher S/N value with the same antibody at its highest concentration.

To develop this platform further, the next section investigates alternative detection methods. Alternative detection may be more compatible with routine testing, and with other tests to be performed on the microarray once a comprehensive blood testing microarray is investigated. For example it may be unpractical to label all blood samples, and target solutions must not cross-react with probes (see Discussion).

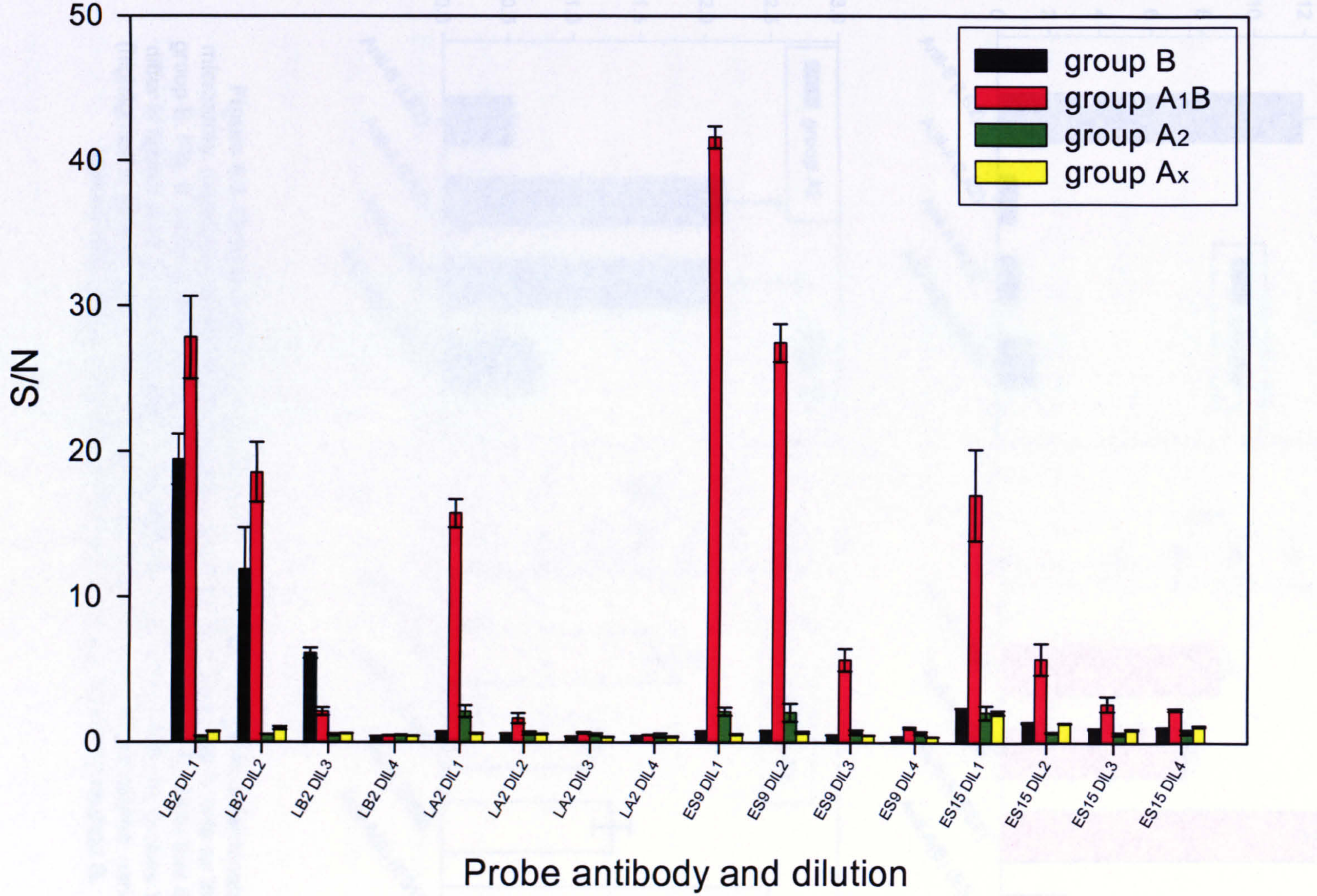
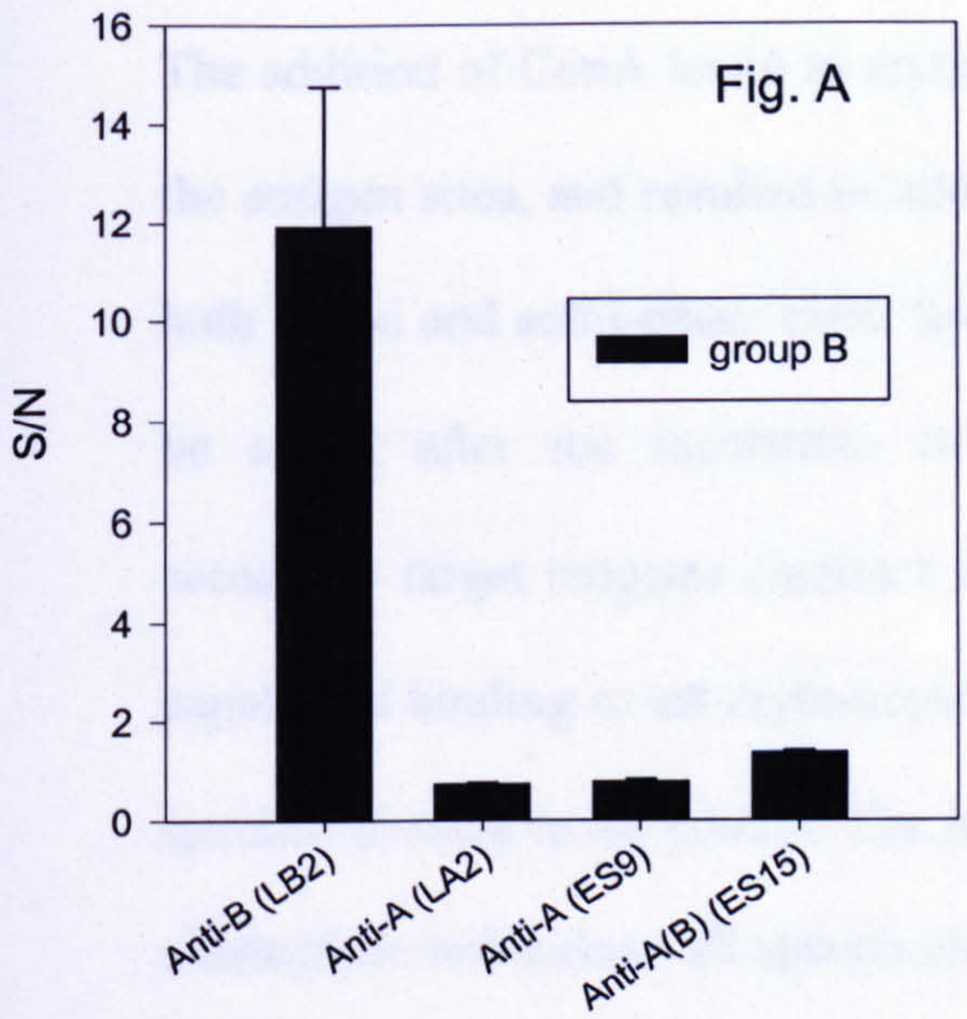
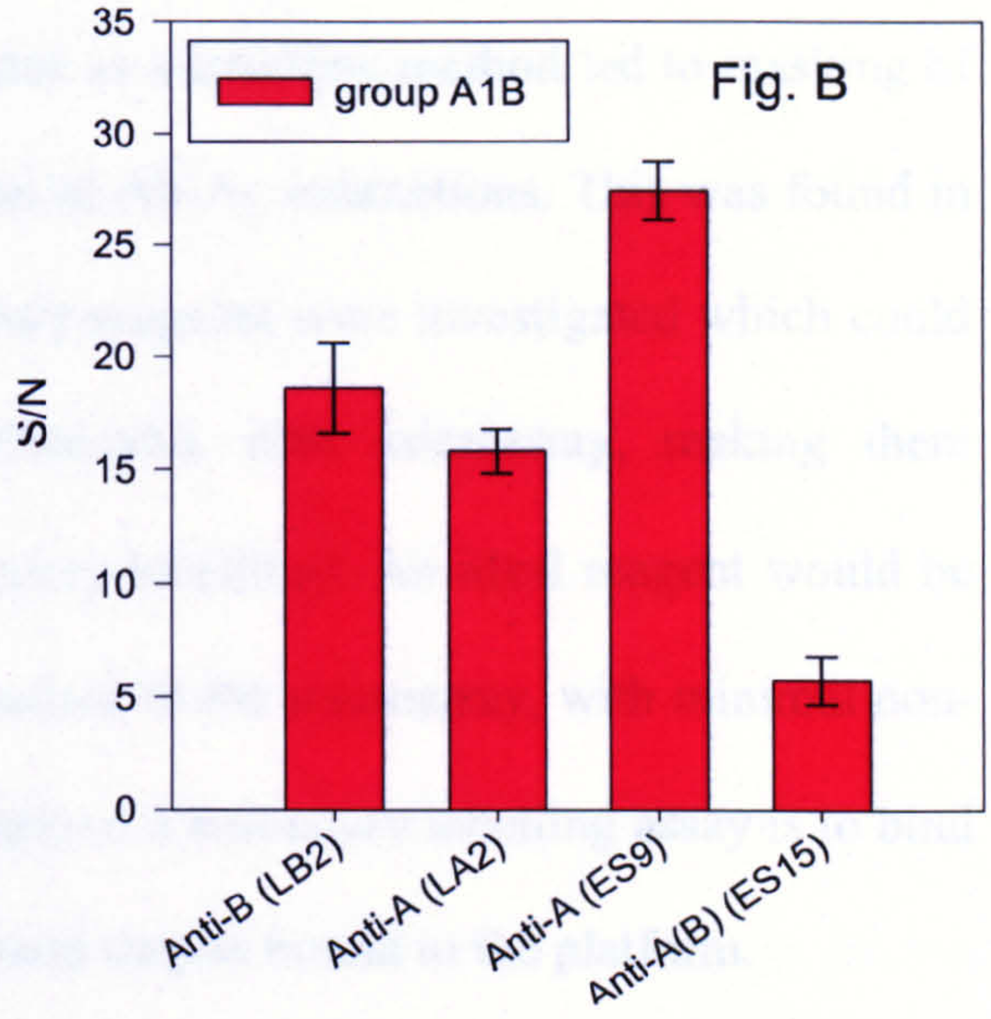
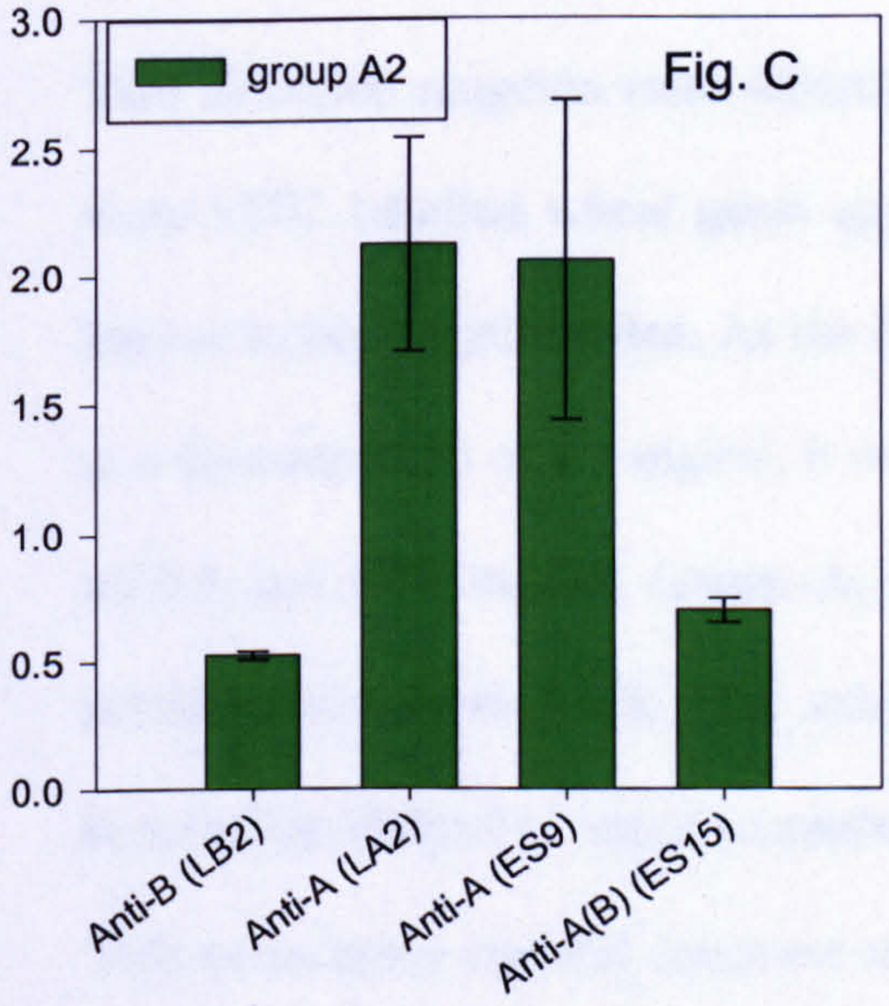



**Figure 4.8.** Detection of reduced expression blood group antigenic determinants by microarray, all used concentrations shown. Slide type poly-L-lysine, slide reps 1, pins 700 $\mu$m, probes Table 4.7, probe reps 3, SPM D; blocker PBS-milk, target/volume: various FITC labeled RBC/450$\mu$l, incubation time 60min, mixing, scanning method B.



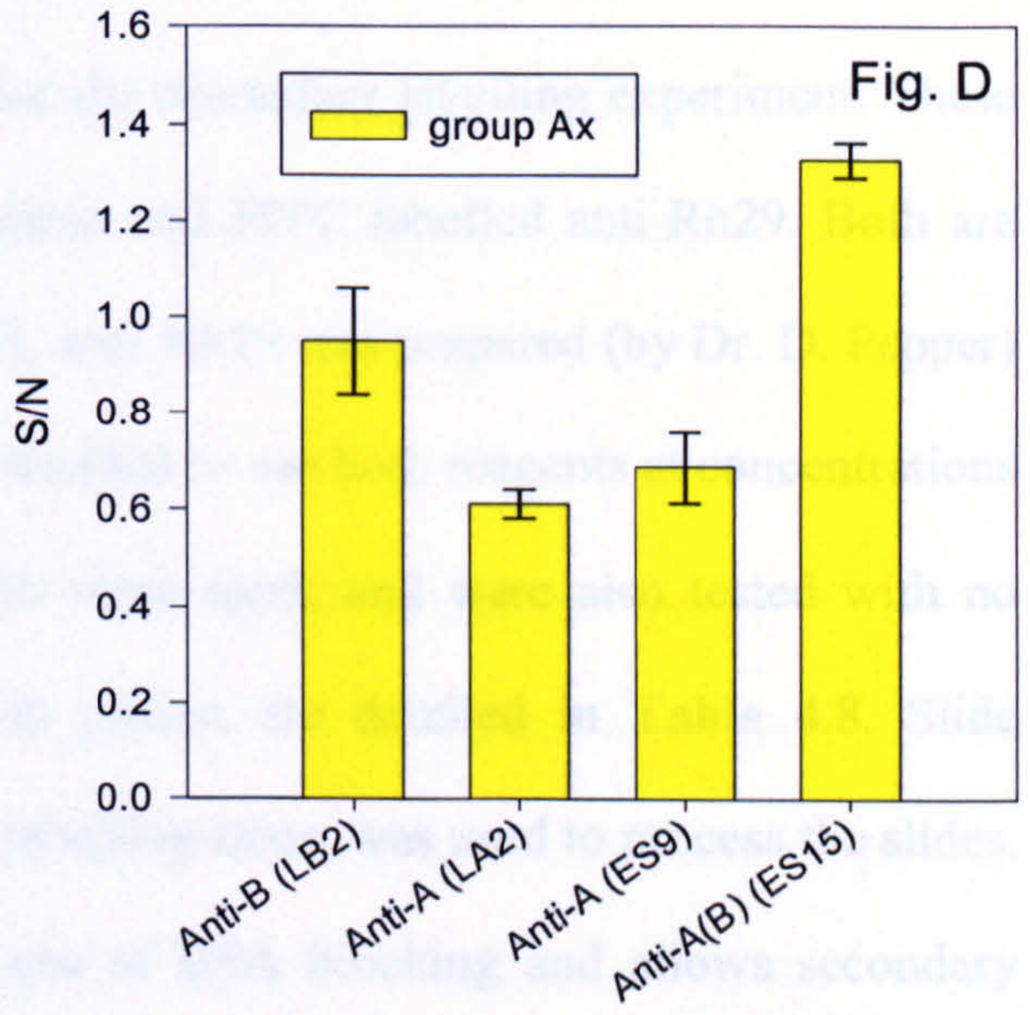




**Figure 4.9.** Detection of reduced expression blood group antigenic determinants by microarray, displaying similar antibody probe concentrations only. Fig A. acts as the control group B. Fig. B shows group $A_1B$, Fig. C group $A_2$, and Fig. D group $A_x$. Note that the scales differ in figures A to D. Slide type poly-L-lysine, slide reps 1, pins 700 $\mu$m, probes Table 4.7 (highlighted in yellow), probe reps 3, SPM D; blocker PBS-milk, target/volume: various FITC labeled RBC/450$\mu$l, incubation time 60min, mixing, scanning method B.

## 4.4 Alternative Detection Methods for Erythrocyte Binding

The addition of ConA lectin to erythrocytes as a labelling method led to masking of the antigen sites, and resulted in inhibition of Ab-Ag interactions. This was found in both liquid and solid-phase tests. Secondary reagents were investigated which could be added after the incubation of erythrocytes with microarray, making them secondary target reagents (indirect secondary labelling). An ideal reagent would be capable of binding to all erythrocytes attached to the microarray, with minimal non-specific binding to the probes. The principle of a secondary labelling assay is to bind a detection molecule to all specifically bound targets bound to the platform.

Two detection reagents were selected for the secondary labelling experiment. These were FITC labelled wheat germ agglutinin and FITC labelled anti-Rh29. Both are known to bind erythrocytes. As the FITC anti-Rh29 was prepared (by Dr. D. Pepper) at a concentration of 0.3 mg/ml, it was decided to use both reagents at concentrations of 0.3 and 0.03 mg/ml. Group $A_1$ cells were used, and were also tested with no labelling to assess NSB. The antibody probes are detailed in **Table 4.8**. Slide Processing Method E, using secondary labelling steps, was used to process the slides. This processing method involves the use of BSA blocking and allows secondary labelling. The change to BSA blocking is explained in Appendix 3.

The results are presented in **Figure 4.10**. Although the signal is lower than that with directly FITC labelled cells, it is still sufficient for specific blood group typing. The effect of autofluorescence of the erythrocytes is seen in **Figure 4.10**. In this slide, the secondary reagent was omitted.

**Table 4.8.** Probes printed onto slides for use in secondary labelling experiment.

| Probe/ Antibody ID | Protein/ Ab type | Conc. DIL1 | Conc. DIL2 |
|---|---|---|---|
| Anti-A (LA2) | mouse IgM | 205 | 50 |
| Anti-B (LB2) | mouse IgM | 484 | 50 |
| Anti-A,(B) ES15 | mouse IgM | 425 | 50 |
| Anti-D (LDM3) | human IgM | 35 | n/a |
| Anti- D (LHM59/19) | human IgG | 1000 | n/a |
| PBS | | n/a | |

For some results, the S/N is higher using autofluorescence than from those obtained with the anti-Rh29. The wheat germ agglutinin gives the optimal results in this experiment, and reacts with higher S/N values when more dilute (at 30 $\mu$g/ml rather than at 300 $\mu$g/ml). Although this area requires further investigation, it shows that there are alternatives to direct labelling. For the purpose of this thesis, work continued using directly labelled erythrocytes.

## 4.5 Alternative Slide Surfaces for Blood Typing Microarrays

As part of the wider group project (Proof of Concept (PoC), see discussion), it was discovered that epoxy silane coated slides gave improved S/N ratios over the poly-L-lysine slides (data not shown). The process had been shown capable of ABO blood grouping, but had not been optimised. Therefore, the blocking was examined in an attempt to improve the S/N values and reduce NSB.

The blocking optimisation was performed as part of this work, and is presented in Appendix 3. The selection of BSA blocking was subsequently applied to gold slides

and was found to give excellent results. This resulted in the fully optimised procedure for processing of slides, which is described as Slide Processing Method E.

## 4.6 Rhesus Typing by Microarray

Mandatory testing of donors and patients includes the determination of RhD blood group status. In the U.K. it is recommended that this testing be carried out using at least two different monoclonal antibodies. If testing donors, partial RhD type VI (DVI) must be detected. Patient testing does not require the detection of DVI (Jones *et al.*, 1995; UK Blood Transfusion Services, 2002). Consequently, antibodies for this purpose must be selected appropriately. It would also be advantageous for a new testing system to incorporate tests for other relevant blood group antigens in addition to mandatory probes. For this reason, available antibodies to Rhesus antigens E and c were also tested.

Monoclonal antibodies for use in the following section were purified by the methods detailed in Chapter 2, where information on all cell lines is presented. All antibodies had been shown to work in haemagglutination assays and the titration end point is also presented in Chapter 2. The identification and concentrations of the extensive range of antibodies used in this section are detailed in **Table 4.9.** All were printed at 1.0 and 0.5 mg/ml and higher, if available, and as detailed on the result figures.

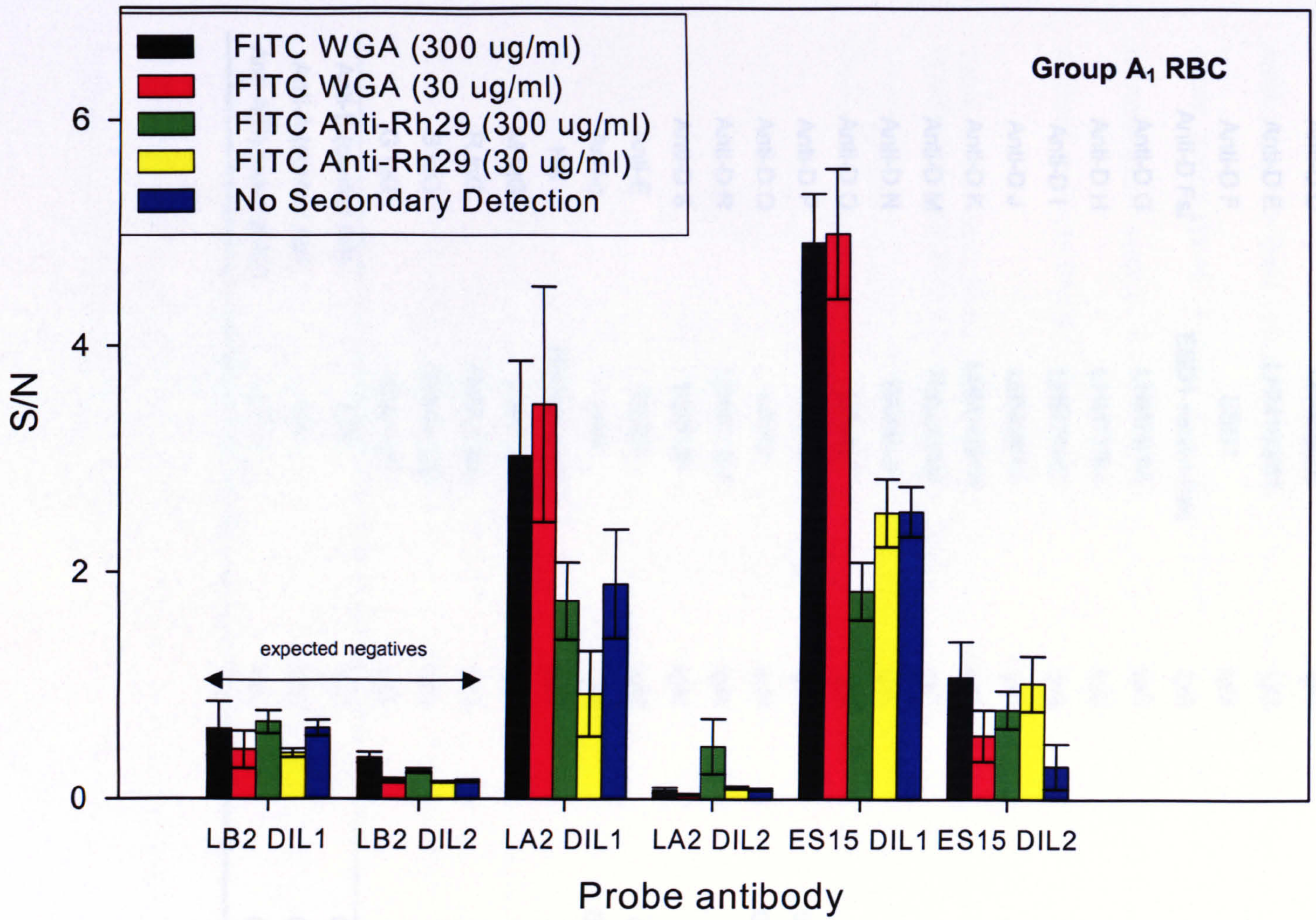



**Figure 4.10**. Results of secondary labelling blood typing experiment.
Slide type poly-L-lysine, slide reps 1, pins 700 $\mu$m, probes Table 4.8, probe reps 8, SPM E; blocker PBS-BSA, target/volume: unlabeled group $A_1$RBC/450$\mu$l, incubation time 60min, mixing, scanning method B.

**Table 4.9.** Probes used in Rhesus blood typing experiments.

| Specificity and identity in results | Cell line (if MAb) or protein identity | Antibody class | Concentration (mg/ml) |
|---|---|---|---|
| Anti-D A | LHM76/58 | IgG | 1.00 |
| Anti-D B | LHM76/59 | IgG | 1.60 |
| Anti-D D | LHM50/2B | IgG | 1.56 |
| Anti-D E | LHM169/81 | IgG | 1.28 |
| Anti-D F | ESD1 | IgG | 1.02 |
| Anti-D Fsf | ESD1 serum free | IgG | 0.29 |
| Anti-D G | LHM76/55 | IgG | 1.78 |
| Anti-D H | LHM77/64 | IgG | 1.07 |
| Anti-D I | LHM70/45 | IgG | 1.38 |
| Anti-D J | LHM59/19 | IgG | 1.07 |
| Anti-D K | LHM169/80 | IgG | 1.09 |
| Anti-D M | Polyclonal | IgG | 2.61 |
| Anti-D N | BRAD-3 | IgG | 1.00 |
| Anti-D O | LDM1 | IgM | 0 |
| Anti-D P | LDM2 | IgM | 0.0087 |
| Anti-D Q | LDM3 | IgM | 0.0348 |
| Anti-D R | LDM77/64 | IgM | 0 |
| Anti-D S | ESD1M | IgM | 0 |
| Anti-E | DEM1 | IgM | 0.437 |
| Anti-c | H48 | IgM | 0.0075 |
| HS | Human serum | n/a | 3 |
| M IgG | LHIVG2 | IgG | 0.3 |
| R IgG | Rabbit IgG | IgG | 0.5 |
| S IgG | Sheep IgG | IgG | 0.5 |
| G IgG | Goat IgG | IgG | 0.5 |
| Anti-B (control Ab) | LB2 | IgM | 0.484 |
| Anti-A (control Ab) | LA2 | IgM | 0.205 |
| Anti-A(B) (control Ab) | ES15 | IgM | 0.425 |

### 4.6.1 Rhesus D, E and c Grouping by Microarray

Antibodies to Rhesus D, E and c were selected as antibody probes for printing onto gold-coated slides. Gold-coated slides have superior properties that could be potentially advantageous for Rhesus grouping. It is probable that gold binds antibodies via thiol groups.The cells selected for use in this experiment showed either homozygous or heterozygous expression of the antigens of interest. The nomenclature used in Rhesus grouping is described in **Table 4.10** and the cells selected are described in **Table 4.11.**

**Table 4.10.** Rhesus nomenclature used in this work, 'antigens present' represents antigens inherited from one parent (review, Daniels, 2002).

| Antigens present | Fisher | Weiner |
|---|---|---|
| C D e | CDe | $R_1$ |
| c D E | cDE | $R_2$ |
| C D E | CDE | $R_z$ |
| c D e | cDe | $R_o$ |
| c d e | cde | r |
| C d e | Cde | r' |
| c d E | cdE | r'' |
| C d E | CdE | $r^y$ |

The cells were selected to demonstrate different antigen site densities of the relevant antigens. For example, a homozygous $R_1R_1$ cell carries two copies of the *RHD* gene and, therefore, expresses a higher amount of RhD antigen, whereas a heterozygous $R_1r$ has only one copy and would be expected to express fewer RhD antigen sites. Homozygous expression was beneficial in this experiment, as antigen site density is higher and should be more easily detected.

Human serum, mouse IgG, rabbit IgG, sheep IgG and goat IgG spots on the microarrays were considered negative controls and were used to calculate noise for the S/N ratios. The results are presented in Figures **4.11a-f.**

**Table 4.11.** Rhesus phenotypes of erythrocyte samples selected for experiment 4.6.1.

| Rhesus phenotype Weiner nomenclature | Rhesus phenotype Fisher-Race nomenclature |
|---|---|
| $R_1R_1$ | CDe/CDe |
| $R_2R_2$ | cDE/cDE |
| $R_1r$ | CDe/cde |
| r'r' | Cde/Cde |
| r''r'' | cdE/cdE |
| rr | cde/cde |

Results from $R_1R_1$ are presented in **Figure 4.11a**. Of the three antigens being tested this cell has only D, and as it presents homozygous expression the antigen density is estimated to be between 14,500 – 19,300 per cell (Rochna and Hughes-Jones, 1965; Hughes-Jones *et al.*, 1971). However, the presence of the C antigen can affect the expression of the D antigen. For example, the $R_2R_2$ phenotype expresses between 15,800-33,300 RhD antigens per cell. The $R_1R_1$ cell reacts clearly with anti-Ds J, K and N with S/N values of up to 28. The highest concentration of antibody does not consistently give the highest S/N value. There is a lower reaction present with anti-D B (S/N 2.3) and reactions with anti-E and anti-c are negative as expected. None of the IgM anti-D react, but this is possibly due to the concentration of antibody or accessibility of antibody to antigen.

**Figure 4.11b** shows the results from the $R_2R_2$ cell. This cell has homozygous expression of D, E and c antigens. According to Hughes-Jones *et al.*, (1971) the cell will express between 15,800-33,300 D sites and 78,000-80,000 c sites. The cell reacts with several of the IgG anti-D and one of the IgM's, as well as a S/N value of 5.3 with the anti-c. No reaction is evident with the anti-E.

The $R_1r$ cell results are shown in **Figure 4.11c**. This cell phenotype has heterozygous expression of D, and therefore has weaker expression than other Rhesus phenotypes at 9,900-14,600 antigen sites per cell (Rochna and Hughes-Jones, 1965; Hughes-Jones *et al.*, 1971). The weaker expression is reflected in the results where the S/N ratios are below eight with the anti-Ds and at a S/N ratio of two with the anti-c. Expression of c is also heterozygous with around half the c antigen sites of a $R_2R_2$ cell (37,000-42,000).

Cells of phenotype r'r' do not express D, E or c antigens on the erythrocyte surface. This cell is effectively a negative control for all three specificities and as seen in **Figure 4.11d**, all S/N values are below 1.2.

Neither r"r" nor rr phenotypes express the D antigen. r"r" cells express both the E and c antigens and in **Figure 4.11e** there are evident interactions with specific antibodies. With the anti-E the reaction is weak at S/N of 3.5, whereas the anti-c reaction is very strong at over 30 S/N. The results of the rr cell are presented in **Figure 4.11f.** This cell should only be positive with anti-c as it has homozygous expression of the c antigen and the results agree with this, giving a S/N ratio of 22.

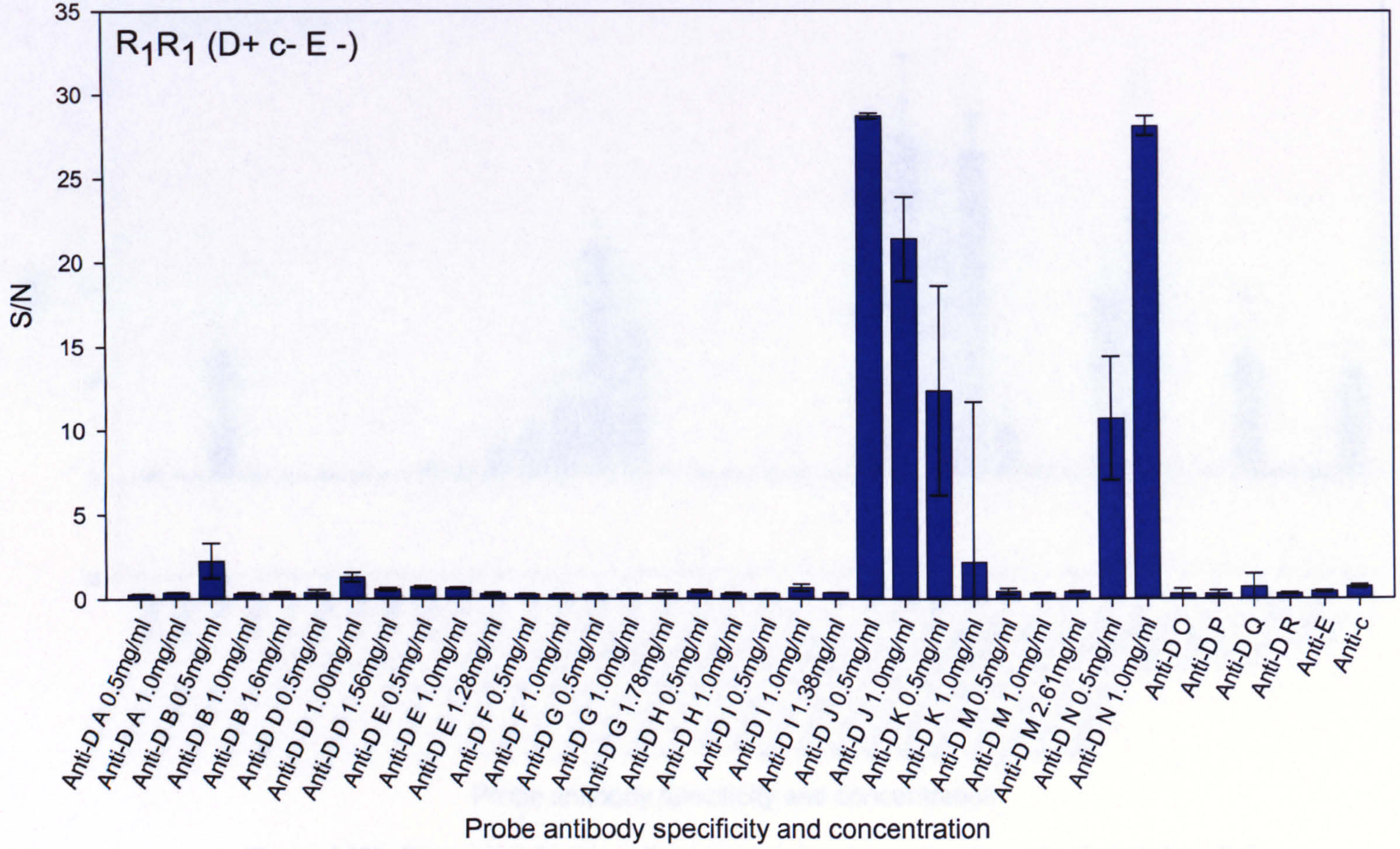



**Figure 4.11a.** Rhesus blood group antigen grouping by microarray, using cells of phenotype $R_1R_1$.
Slide type gold, slide reps 1, pins 700 $\mu$m, probes Table 4.9, probe reps 3, SPM E; blocker PBS-BSA, target/volume: FITC group $R_1R_1$/450$\mu$l, incubation time 60min, mixing, scanning method B.

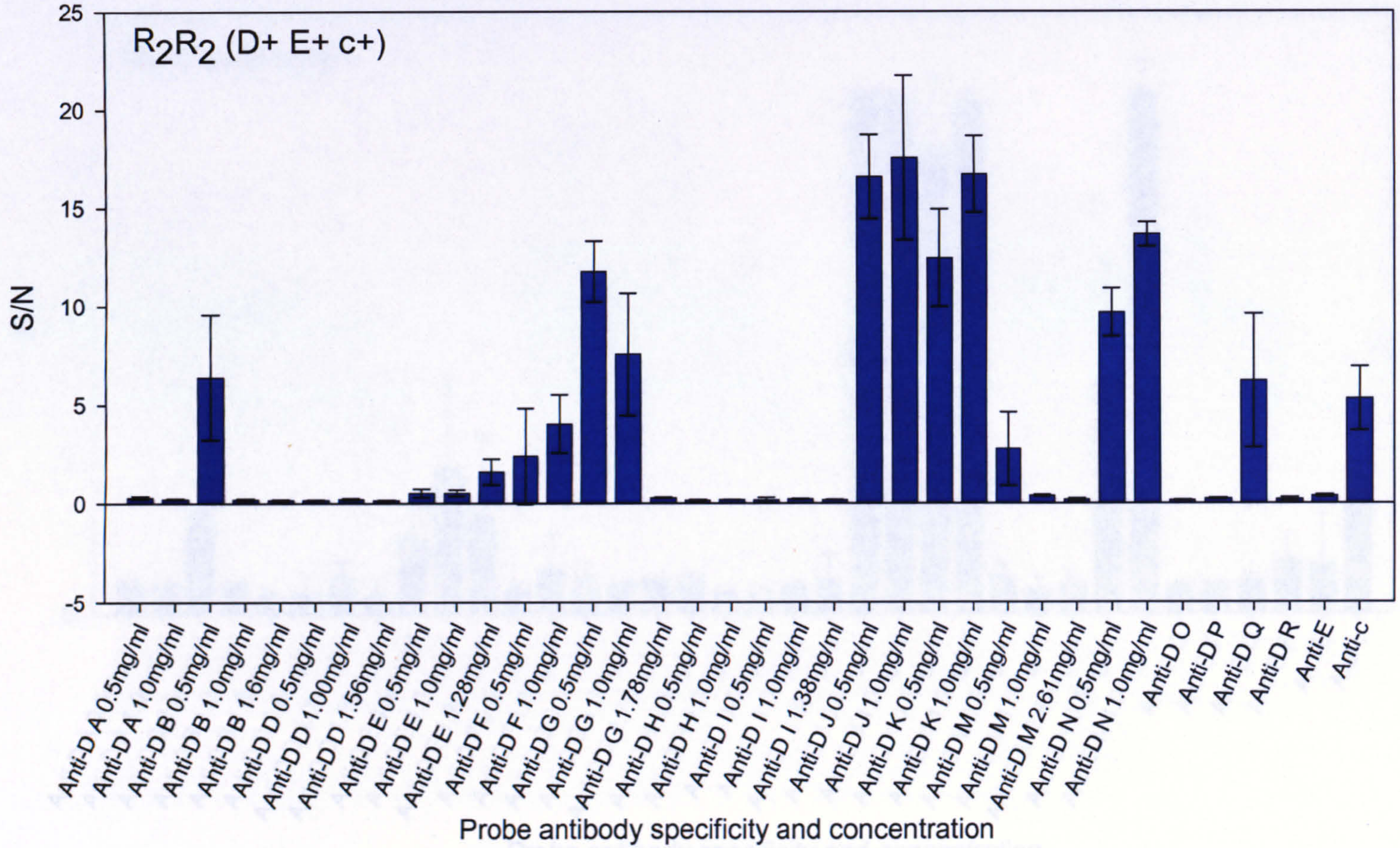



**Figure 4.11b.** Rhesus blood group antigen grouping by microarray, using cells of phenotype $R_2R_2$.
Slide type gold, slide reps 1, pins 700 $\mu$m, probes Table 4.9, probe reps 3, SPM E; blocker PBS-BSA, target/volume: FITC group $R_2R_2$/450$\mu$l, incubation time 60min, mixing, scanning method B.

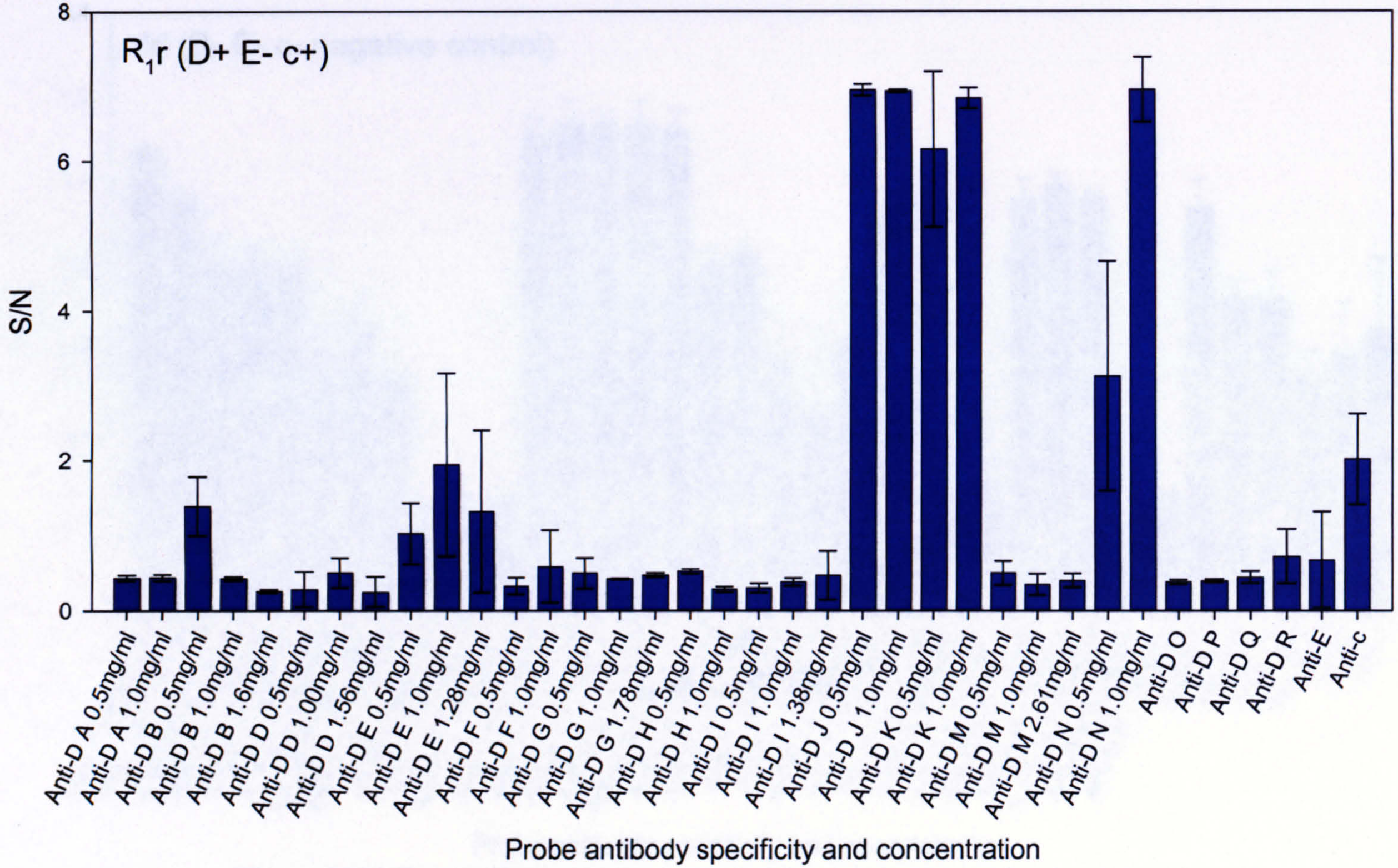



**Figure 4.11c.** Rhesus blood group antigen grouping by microarray, using cells of phenotype $R_1r$.
Slide type gold, slide reps 1, pins 700 $\mu$m, probes Table 4.9, probe reps 3, SPM E; blocker PBS-BSA, target/volume: FITC group $R_1r$/450$\mu$l, incubation time 60min, mixing, scanning method B.

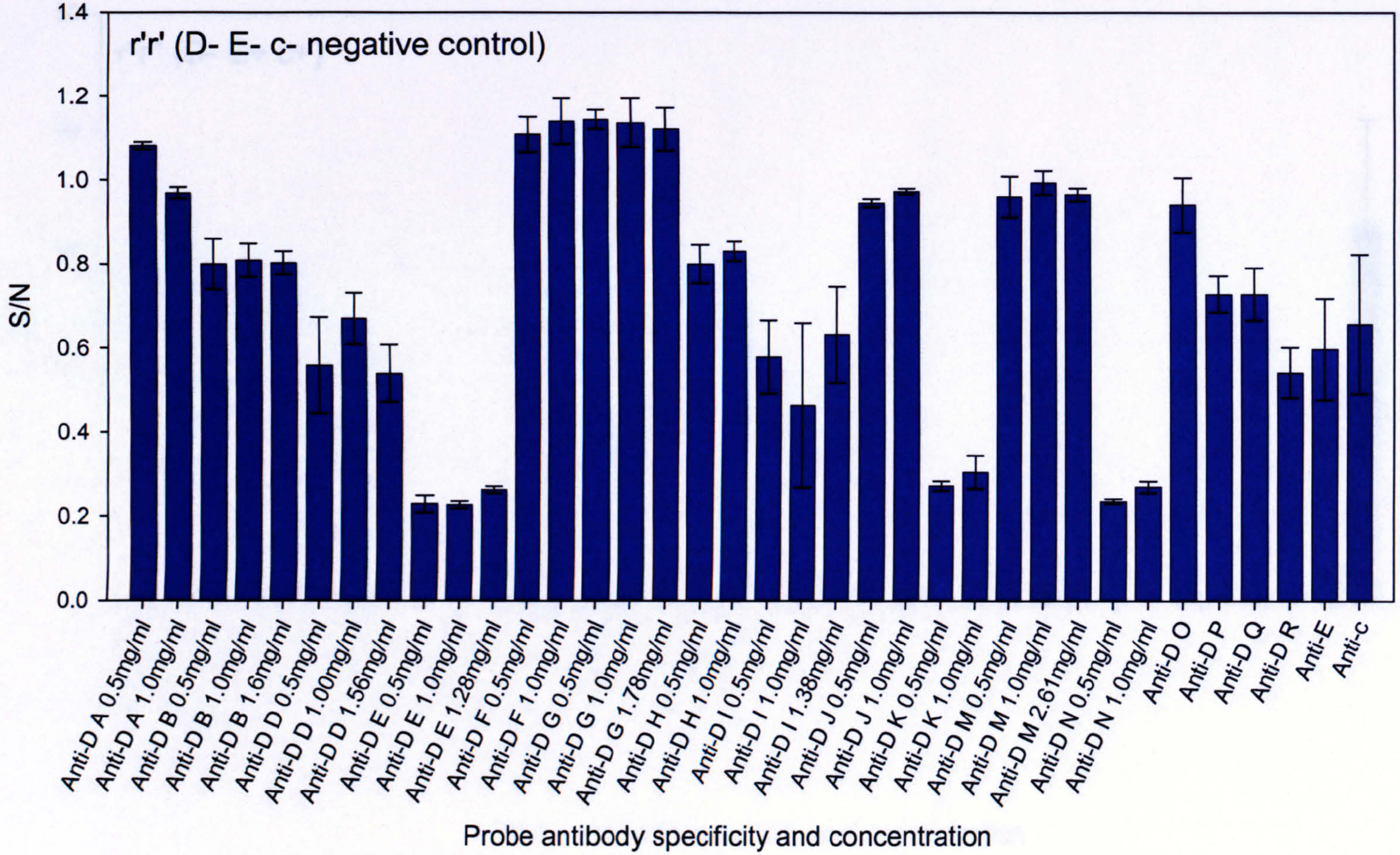



**Figure 4.11d.** Rhesus blood group antigen grouping by microarray, using cells of phenotype r'r'.
Slide type gold, slide reps 1, pins 700 $\mu$m, probes Table 4.9, probe reps 3, SPM E; blocker PBS-BSA, target/volume: FITC group r'r'/450$\mu$l, incubation time 60min, mixing, scanning method B.

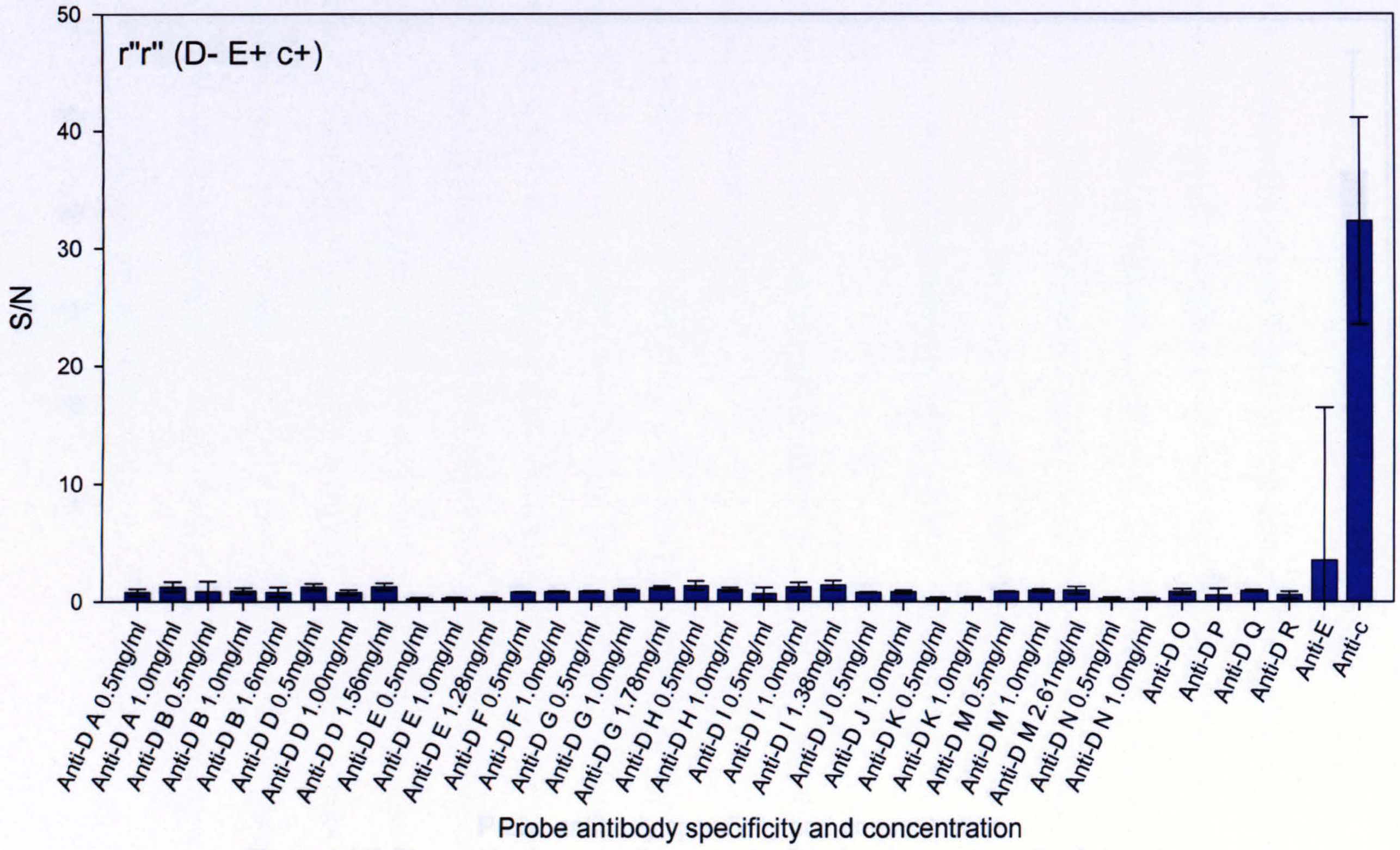



**Figure 4.11e.** Rhesus blood group antigen grouping by microarray, using cells of phenotype r''r''.
Slide type gold, slide reps 1, pins 700 $\mu$m, probes Table 4.9, probe reps 3, SPM E; blocker PBS-BSA, target/volume: FITC group r''r''/450$\mu$l, incubation time 60min, mixing, scanning method B.

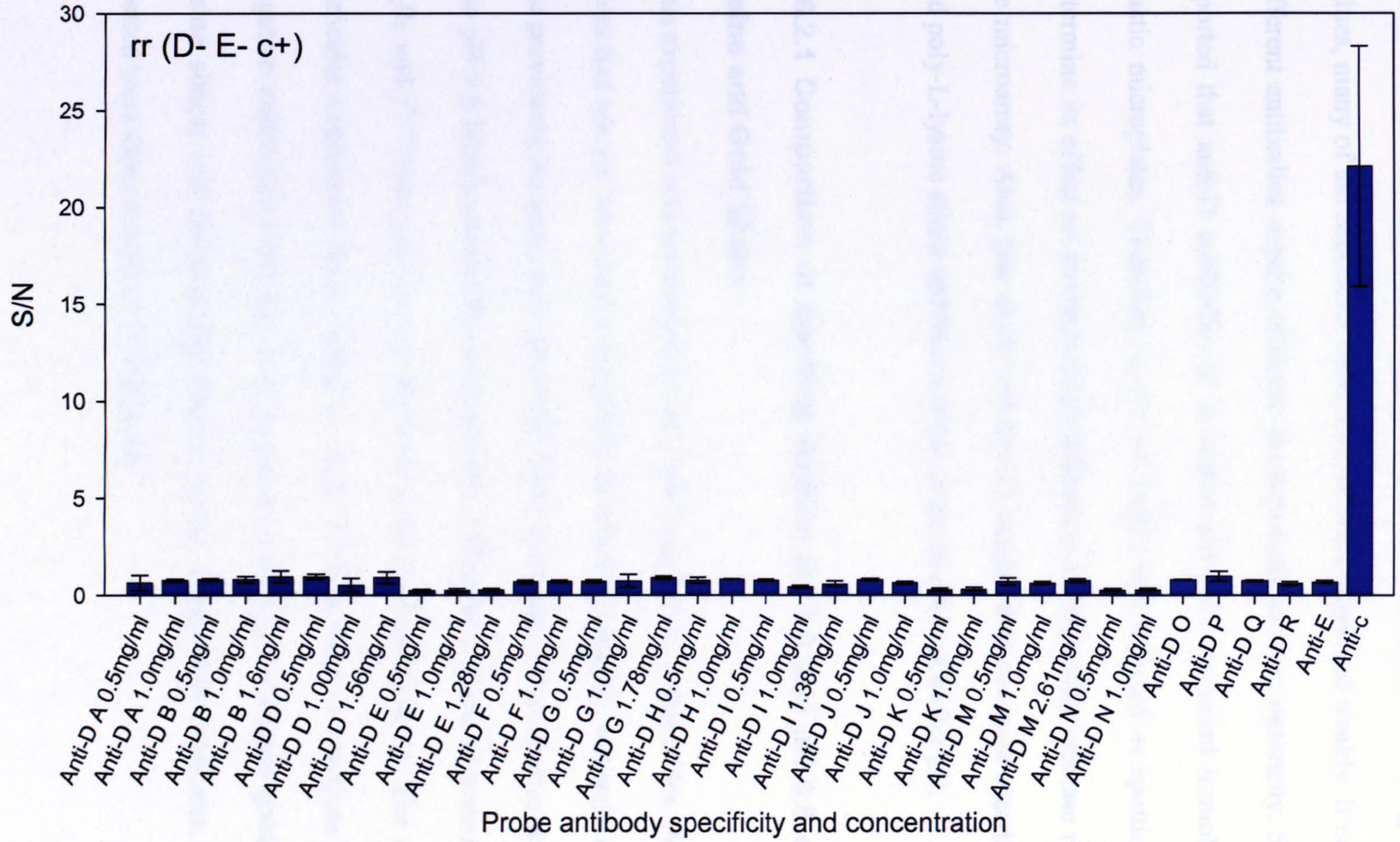



**Figure 4.11f.** Rhesus blood group antigen grouping by microarray, using cells of phenotype rr.
Slide type gold, slide reps 1, pins 700 $\mu$m, probes Table 4.9, probe reps 3, SPM E; blocker PBS-BSA, target/volume: FITC group rr/450$\mu$l, incubation time 60min, mixing, scanning method B.

### 4.6.2 The Effect of pH on Probe Immobilisation and Performance

Rhesus blood typing by microarray was performed with successful results in the previous section. However, although some antibodies bound cells to give high S/N values, many of the antibodies either did not react or reacted weakly. It is known that different antibodies require different environments to react optimally. Scott (1991) reported that anti-D antibodies at increased pH gave increased immobilisation to plastic microplates. Therefore, a pH 9.6 buffer was selected as spotting buffer to determine its effect on antibody immobilisation and hopefully increase reactivity on the microarray. Also, this work was further extended to compare reactions on gold and poly-L-lysine slides and then a wider range of spotting buffer pH.

#### 4.6.2.1 Comparison of Spotting Buffers pH 7.0 and pH 9.6 on Poly-L-lysine and Gold Slides

This experiment was performed on both gold and poly-L-lysine slides. Poly-L-lysine slides had not yet been used successfully for Rhesus grouping, but were used as they had previously demonstrated successful ABO grouping. All antibodies were dialysed into pH 9.6 bicarbonate buffer as described in Chapter 2. Cells of phenotypes $R_1R_1$, $R_2R_2$ and r"r" were used and slides were performed in triplicate. The aims of this particular experiment were, therefore, three-fold: to improve reactions of weak or negative antibodies from previous experiment in 4.6.1; compare gold to poly-L-lysine slides; and demonstrate Rhesus typing using slide replicates. Antibodies spotted were those described in **Table 4.9.**

The $R_1R_1$ reactivity in **Figure 4.12a** is improved from that in **Figure 4.11a.** The S/N values in general are far higher on gold slides, most likely as background noise values were lower. This may also be due to a different $R_1R_1$ cell being used, and the variance that can be apparent from expression levels in different individuals. The error bars show the variability between the three slide replicates. It is very clear that the gold slides give far higher S/N values than the poly-L-lysine slides. In the previous experiment, $R_1R_1$ cells reacted with anti-Ds B, J, K and N. In this experiment an increased number of anti-D are reacting. Anti-Ds A, B and D are giving low reactivity at pH 9.6. Anti-D F and G, which did not react before, are giving S/N values of 41 and 14 respectively at pH 7.0. As before, anti-Ds J, K and N are all reacting well at pH 7.0.

**Figure 4.12b** shows the results of the $R_2R_2$ testing. Again S/N values are generally higher than those found in the previous experiment. The S/N values are far higher on the gold slides. However, the anti-E and anti-c do not react at all. In **Figure 4.12b** in the previous experiment only the anti-c reacted weakly.

In **Figure 4.12c,** the r''r'' cell is showing some false positive results with some of the anti-Ds, especially those at pH 9.6. However, it is encouraging that the three 'best' performing anti-D, J, K and N, are not giving high value non-specific reactions. The anti-E and anti-c look specific but the S/N values are far lower than some of those from the non-specific anti-D reactions.

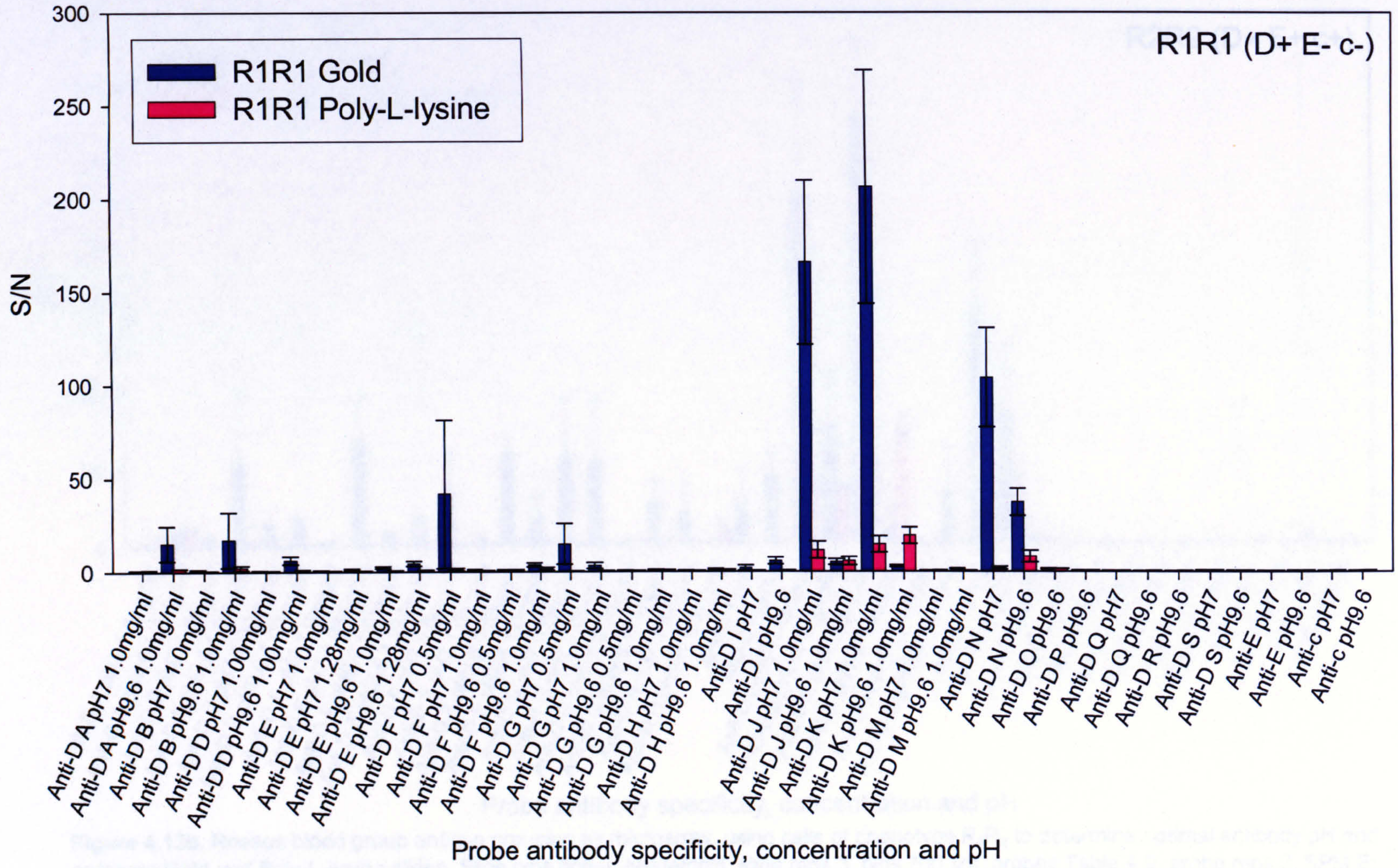



**Figure 4.12a.** Rhesus blood group antigen grouping by microarray, using cells of phenotype $R_1R_1$ to determine optimal antibody pH and compare Gold and Poly-L-lysine slides. Slide type poly-L-lysine/gold, slide reps 3, pins 700 $\mu$m, probes Table 4.9, probe reps 2, SPM E; blocker PBS-BSA, target/volume: FITC group $R_1R_1$/450$\mu$l, incubation time 60min, mixing, scanning method B.

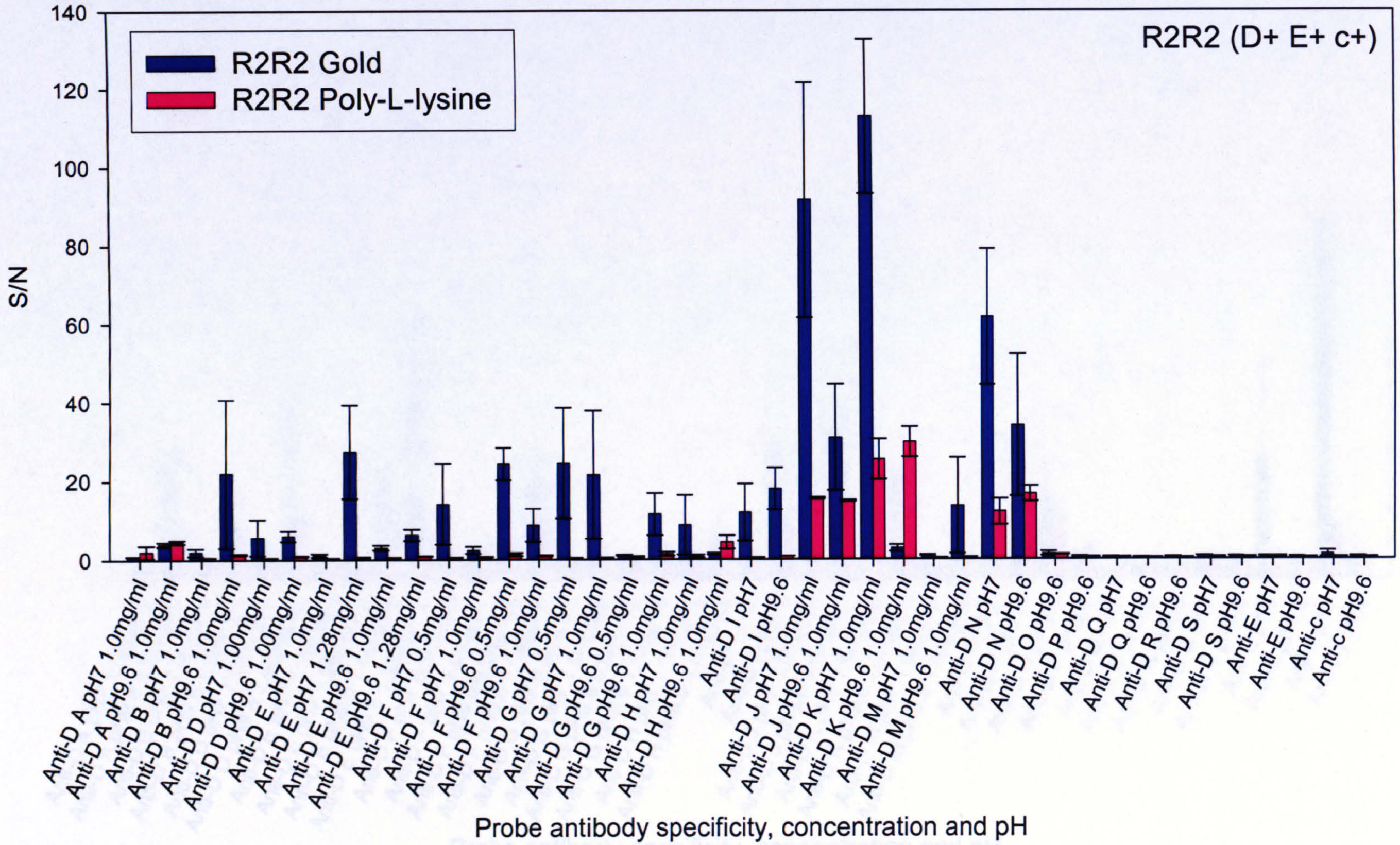



**Figure 4.12b.** Rhesus blood group antigen grouping by microarray, using cells of phenotype $R_2R_2$ to determine optimal antibody pH and compare Gold and Poly-L-lysine slides. Slide type poly-L-lysine/gold, slide reps 3, pins 700 $\mu$m, probes Table 4.9, probe reps 2, SPM E; blocker PBS-BSA, target/volume: FITC group $R_2R_2$/450$\mu$l, incubation time 60min, mixing, scanning method B.

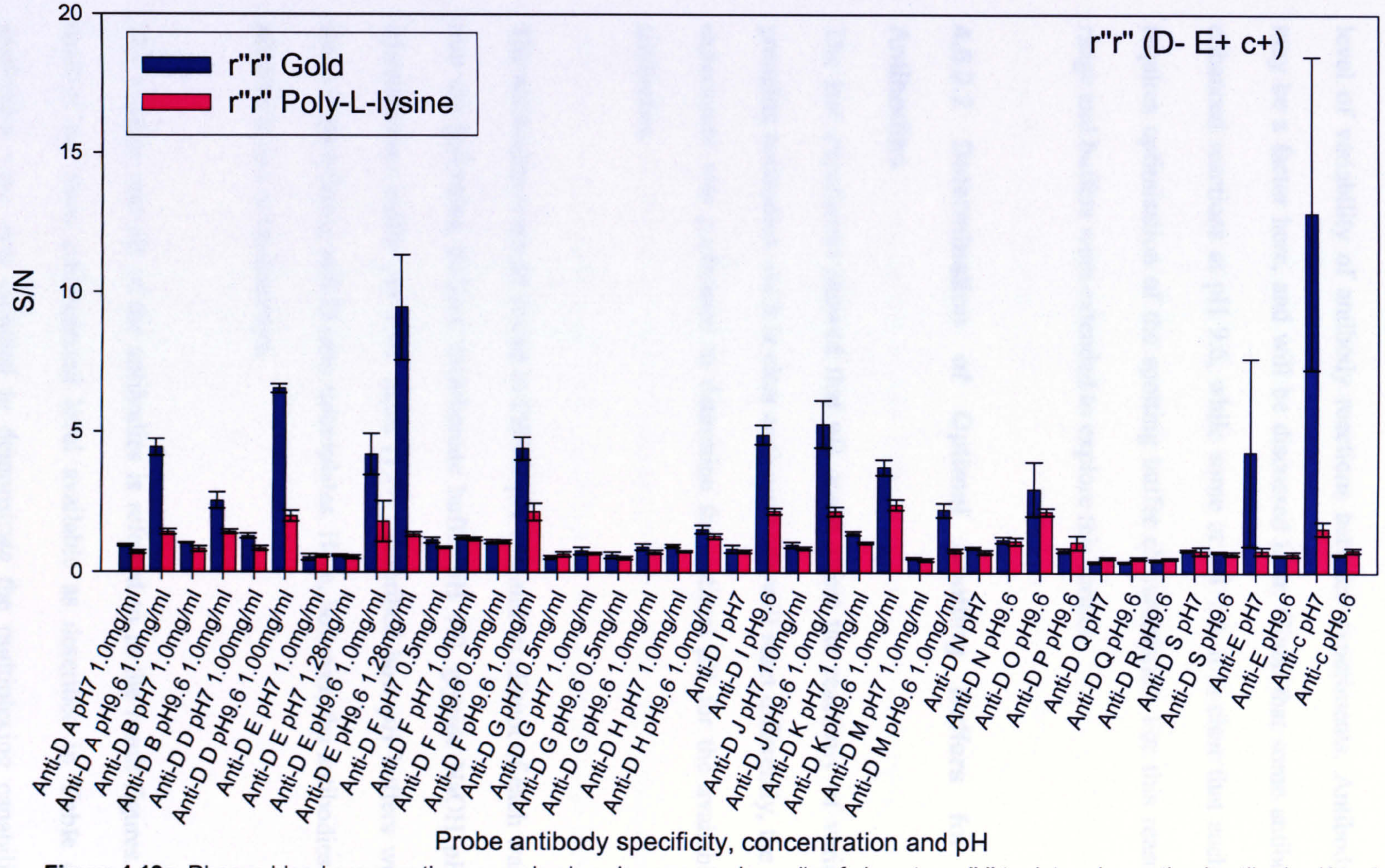



**Figure 4.12c.** Rhesus blood group antigen grouping by microarray, using cells of phenotype r"r" to determine optimal antibody pH and compare Gold and Poly-L-lysine slides. Slide type poly-L-lysine/gold, slide reps 3, pins 700 $\mu$m, probes Table 4.9, probe reps 2, SPM E; blocker PBS-BSA, target/volume: FITC group r"r"/450$\mu$l, incubation time 60min, mixing, scanning method B.

This experiment has demonstrated that gold slides give greater S/N values than the poly-L-lysine slides, that some anti-D react better at higher pH, and that there is a level of variability of antibody reactions between experiments. Antibody stability may be a factor here, and will be discussed later. Given that some antibodies give enhanced reactions at pH 9.6, while some at pH 7.0, it is clear that each antibody requires optimisation of the spotting buffer characteristics. For this reason the pH range and buffers were extended to explore this further.

### 4.6.2.2 Determination of Optimal Spotting Buffers for Rhesus Antibodies

The last experiment showed that pH could affect the reactivity of various blood grouping antibodies. As it is clear each antibody will react differently, the following experiment was performed to determine the optimal pH for the available Rhesus antibodies.

The antibodies were all stored in PBS at pH 7.0, and an aliquot of each was dialysed into the following buffers; bicarbonate buffer pH 9.0, glycine NaOH pH 9.8 and ethanolamine buffer pH 10.4. Scott (1991) described how pH buffers were useful when immobilising anti-D onto microplates. Here, however, the antibodies are being adapted to use in microarrays.

The identity and pH of the antibodies is referred to in the result figures. All were used at the most concentrated level available as described in **Table 4.9.** ABO antibodies were also included to demonstrate the multiplexing capability of the

microarray, but these were used only at pH 7.0. Samples were printed in duplicate onto gold-coated slides. FITC labelled erythrocyte samples were selected which express various ABO and Rhesus groups, details of which are in the figures. The results of this experiment are presented in **Figures 4.13a-g**. Due to the large amount of data generated in this experiment, the results presented are from those probe antibodies which gave S/N values of greater than one, and are arranged in descending order. With results presented in this format, the optimal antibody and pH can be seen more clearly.

A group B $R_2r$ erythrocyte sample was evaluated on the microarray. The results in **Figure 4.13a** confirm that this cell is a group B as it reacts strongly with anti-B LB2, and RhD positive due to its reactions with several anti-D.

**Figure 4.13b** shows the results from a group O $R_1R_2$ cell, which gives excellent S/N ratios with anti-D probes. Anti-Ds K and N react well at pH 7.0 while anti-D J gives a positive reaction at all pH except 9.0.

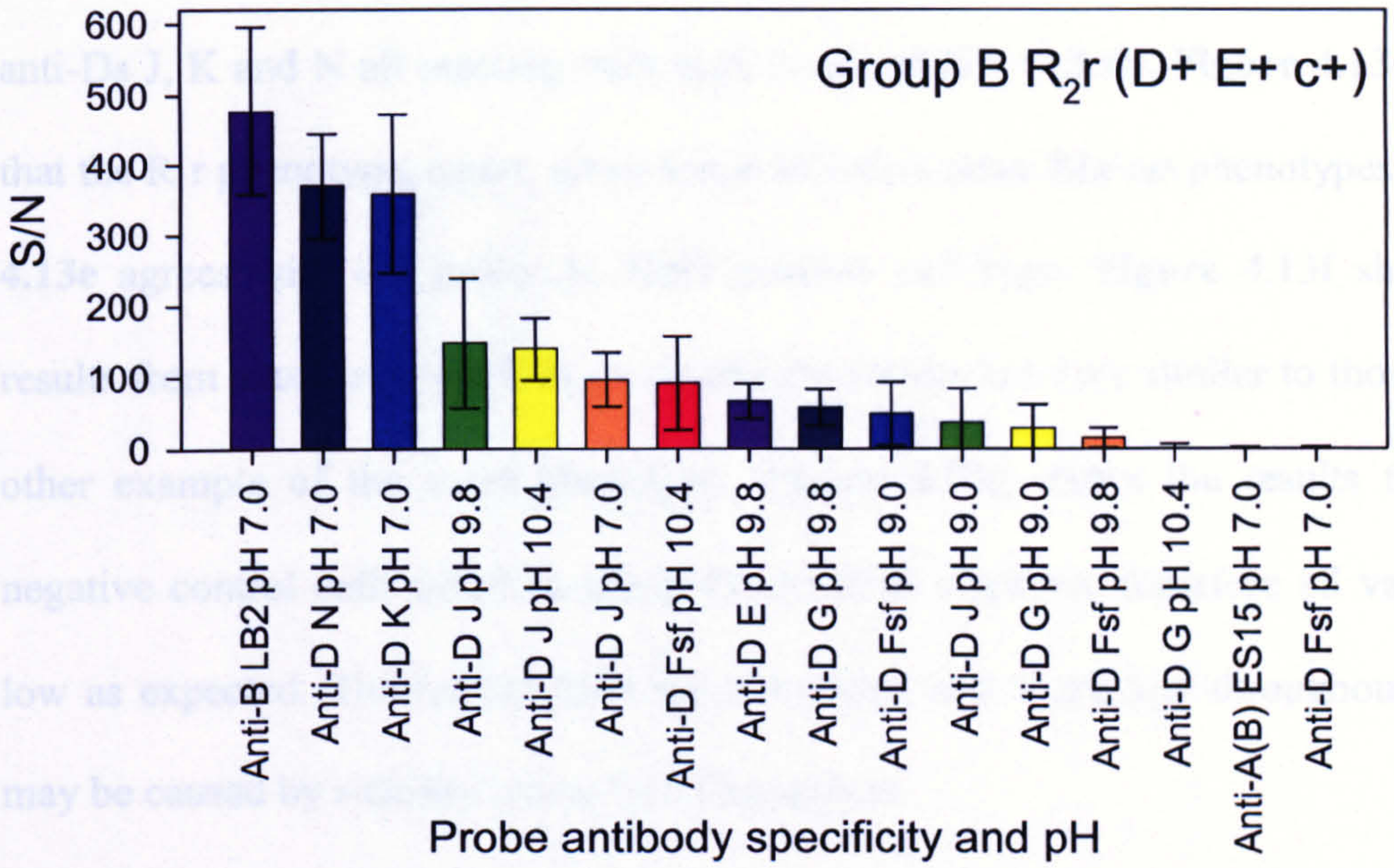



**Figure 4.13a**. Optimal spotting buffer pH determination, using group B $R_2r$ cell. Slide type gold, slide reps 3, pins 700 $\mu$m, probes Table 4.9, probe reps 2, SPM E; blocker PBS-BSA, target/volume: FITC group B $R_2r$/450$\mu$l, incubation time 60min, mixing, scanning method B.

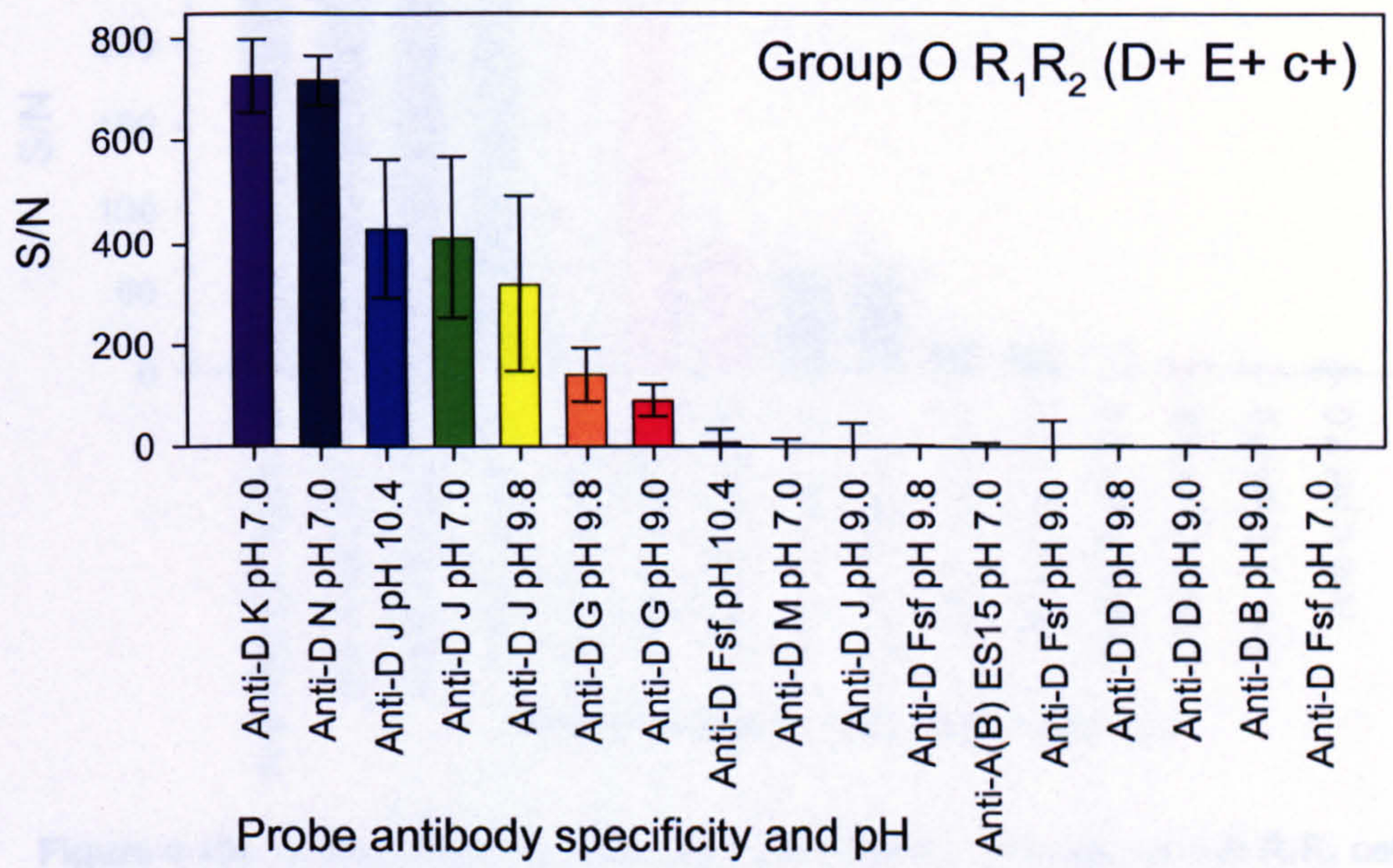



**Figure 4.13b**. Optimal spotting buffer pH determination, using group O $R_1R_2$ cell. Slide type gold, slide reps 3, pins 700 $\mu$m, probes Table 4.9, probe reps 2, SPM E; blocker PBS-BSA, target/volume: FITC group O $R_1R_2$/450$\mu$l, incubation time 60min, mixing, scanning method B.

**Figure 4.13c** confirms the group of the $A_1B$ RhD positive cell tested, again with anti-Ds J, K and N all reacting with high levels of S/N (>200). **Figure 4.13d** shows that the $R_1r$ phenotype, again, gives lower S/N than other Rhesus phenotypes. **Figure 4.13e** agrees with the group $A_1$ RhD positive cell type**. Figure 4.13f** shows the results from another group O $R_1r$ cell and the results are very similar to those of the other example of the same phenotype. **Figure 4.13g** shows the results from the negative control cell, which is group O and RhD negative, therefore all values are low as expected. The results from the anti-c and anti-E are low throughout, which may be caused by stability issues (see discussion).

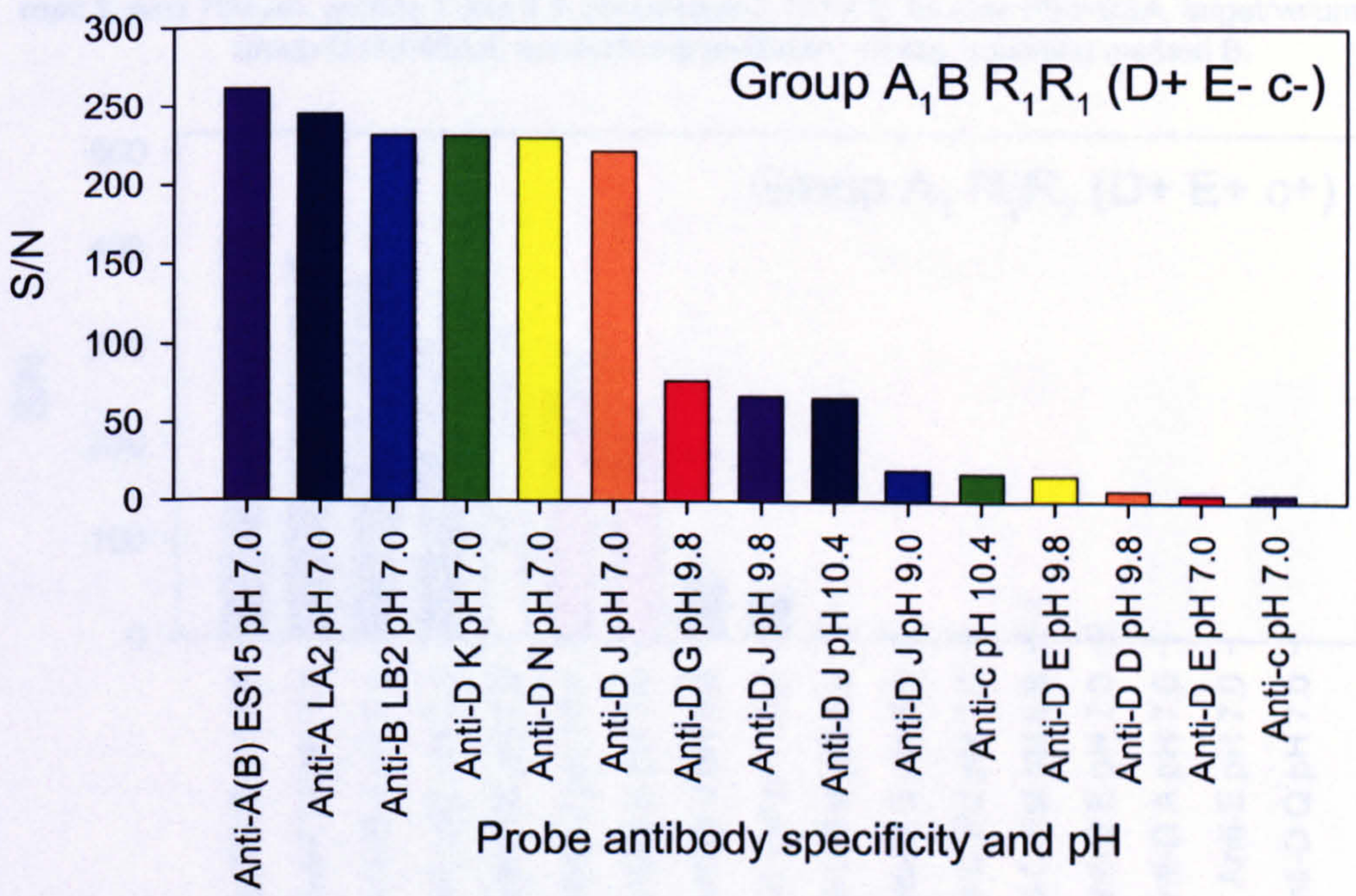



**Figure 4.13c**. Optimal spotting buffer pH determination, using group $A_1B$ $R_1R_1$ cell. There are no error bars as only one slide performed for this cell. Slide type gold, slide reps 1, pins 700 $\mu$m, probes Table 4.9, probe reps 2, SPM E; blocker PBS-BSA, target/volume: FITC group $A_1B$ $R_1R_1$ /450$\mu$l, incubation time 60min, mixing, scanning method B.

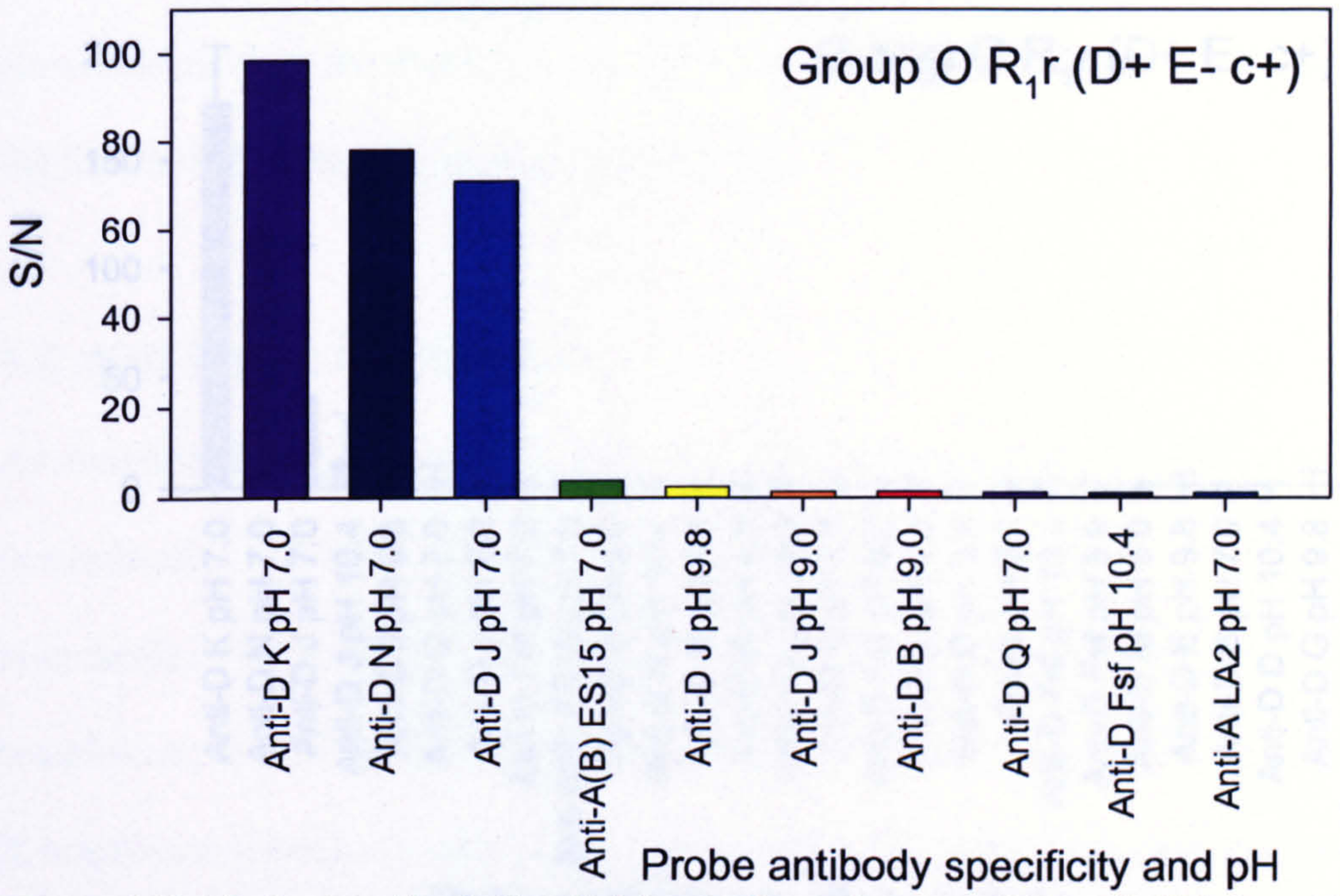



**Figure 4.13d**. Optimal spotting buffer pH determination, using group O $R_1r$ cell. Slide type gold, slide reps 1, pins 700 $\mu$m, probes Table 4.9, probe reps 2, SPM E; blocker PBS-BSA, target/volume: FITC group O $R_1r$/450$\mu$l, incubation time 60min, mixing, scanning method B.

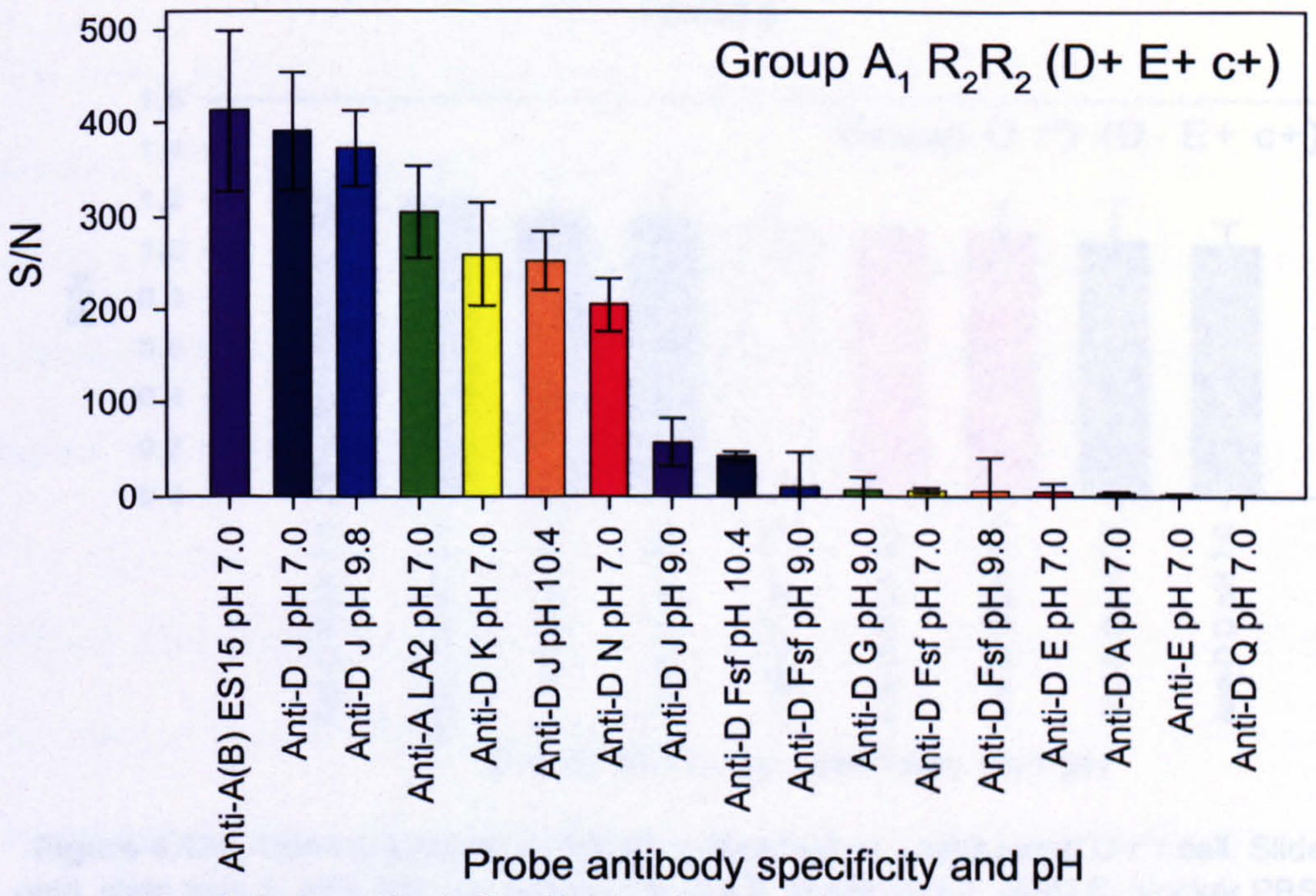



**Figure 4.13e**. Optimal spotting buffer pH determination, using group $A_1$ $R_2R_2$ cell. Slide type gold, slide reps 3, pins 700 $\mu$m, probes Table 4.9, probe reps 2, SPM E; blocker PBS-BSA, target/volume: FITC group $A_1$ $R_2R_2$/450$\mu$l, incubation time 60min, mixing, scanning method B.

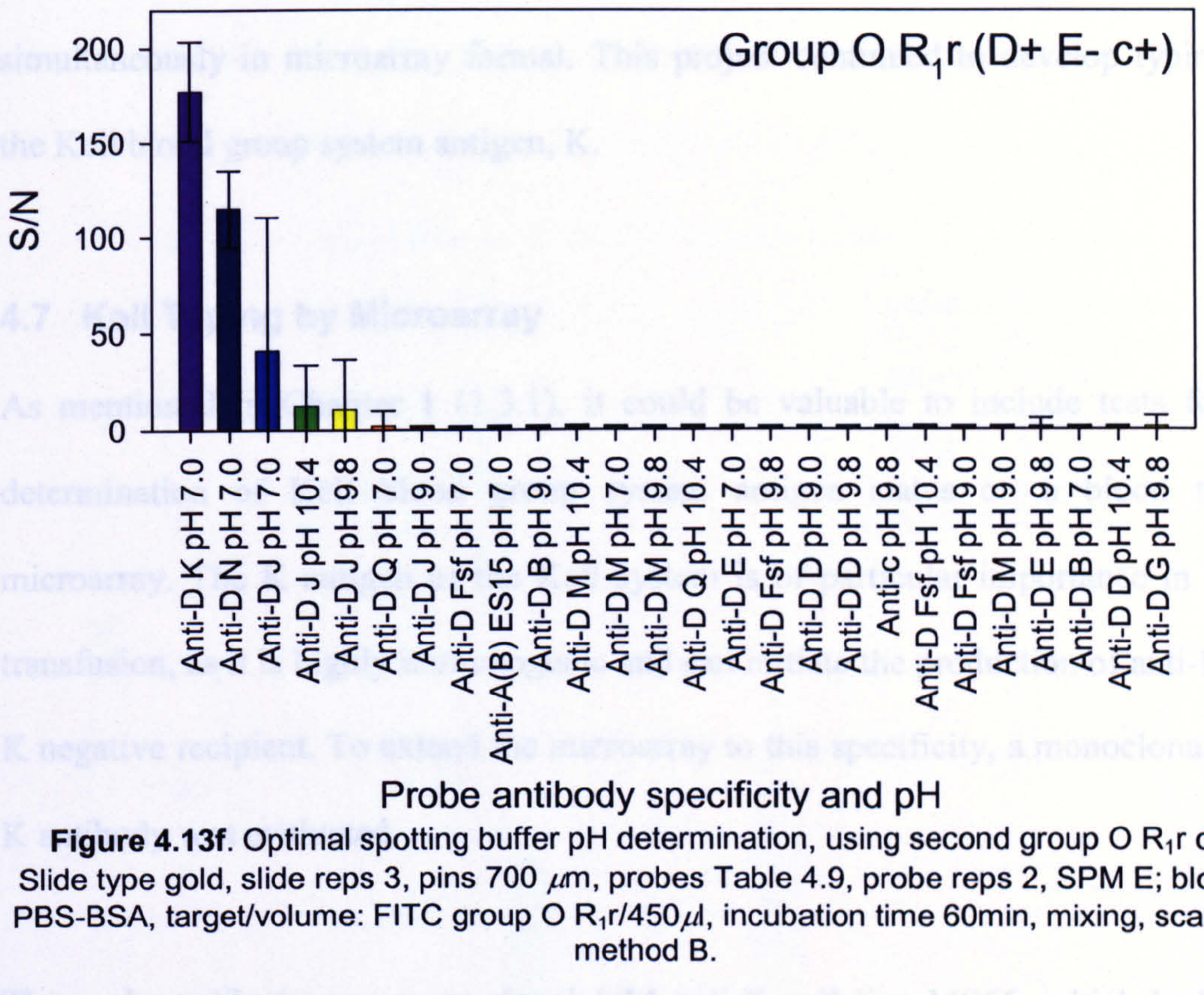



**Figure 4.13f**. Optimal spotting buffer pH determination, using second group O $R_1r$ cell. Slide type gold, slide reps 3, pins 700 $\mu$m, probes Table 4.9, probe reps 2, SPM E; blocker PBS-BSA, target/volume: FITC group O $R_1r$/450$\mu$l, incubation time 60min, mixing, scanning method B.

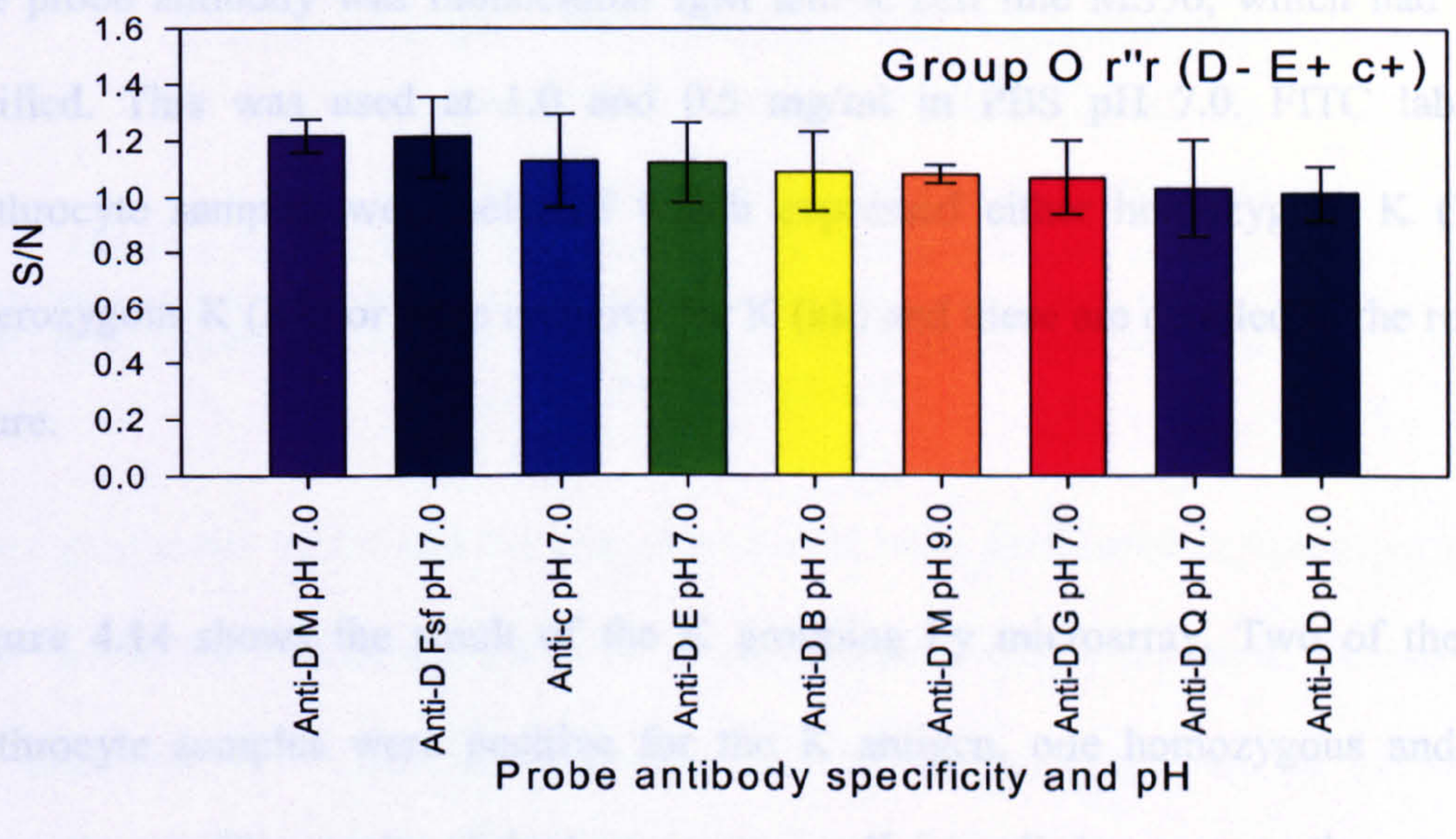



**Figure 4.13g**. Optimal spotting buffer pH determination, using group O r''r cell. Slide type gold, slide reps 3, pins 700 $\mu$m, probes Table 4.9, probe reps 2, SPM E; blocker PBS-BSA, target/volume: FITC group O r''r/450$\mu$l, incubation time 60min, mixing, scanning method B.

This section concluded that ABO and Rhesus typing can be performed simultaneously in microarray format. This project continued to develop typing for the Kell blood group system antigen, K.

## 4.7 Kell Typing by Microarray

As mentioned in Chapter 1 (1.3.1), it could be valuable to include tests for the determination of Kell blood group system antigen status on a blood typing microarray. The K antigen of the Kell system is of particular importance in blood transfusion, as it is highly immunogenic and can initiate the production of anti-K in a K negative recipient. To extend the microarray to this specificity, a monoclonal anti-K antibody was evaluated.

The probe antibody was monoclonal IgM anti-K cell line MS56, which had been purified. This was used at 1.0 and 0.5 mg/ml in PBS pH 7.0. FITC labelled erythrocyte samples were selected which expressed either homozygous K (KK), heterozygous K (Kk) or were negative for K (kk) and these are detailed in the results figure.

**Figure 4.14** shows the result of the K grouping by microarray. Two of the test erythrocyte samples were positive for the K antigen, one homozygous and one heterozygous. The results of the homozygous cell (in red) demonstrate the expected results i.e. higher with the more concentrated probe antibody spots. The heterozygous cell fails to react with the 1.0 mg/ml spot of antibody. The 1.0 mg/ml

spots also give a false positive reaction with a K negative cell (in green). It was concluded that the 0.5 mg/ml probe antibody concentration is optimal.

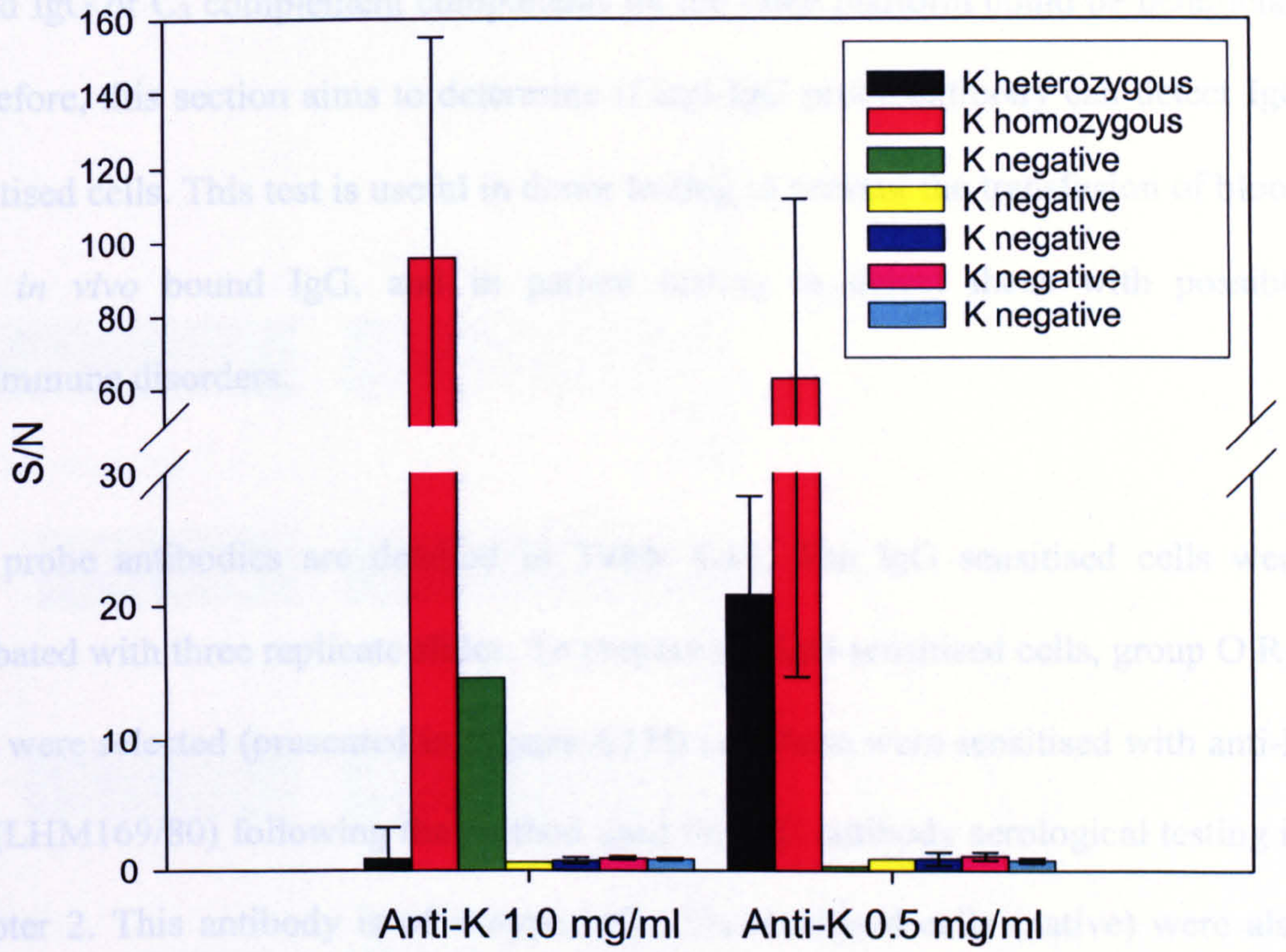



**Figure 4.14.** K grouping by microarray, using cells of varying K antigen status. Slide type gold, slide reps 3, pins 700 $\mu$m, probes anti-K only, probe reps 2, SPM E; blocker PBS-BSA, target/volume: FITC labelled various RBC/450$\mu$l, incubation time 60min, mixing, scanning method B.

The experiments in this and the previous section were performed as one experiment, with the results presented separately for clarification. This experiment demonstrates for the first time that ABO, Rhesus and K grouping can all be performed simultaneously in an open-plan blood typing microarray. This also demonstrates the capability and potential for multiplex analysis, as different types of antigen can be detected in one experiment i.e. carbohydrate (ABO) and protein (Rhesus, K).

## 4.8 Direct Antiglobulin Test by Microarray

This project has shown successfully that the mandatory blood typing can be performed on a blood typing microarray platform. The inclusion of a test for *in vivo* bound IgG or $C_3$ complement components on the same platform could be beneficial. Therefore, this section aims to determine if anti-IgG probe antibody can detect IgG sensitised cells. This test is useful in donor testing to prevent the transfusion of blood with *in vivo* bound IgG, and in patient testing to detect those with possible autoimmune disorders.

The probe antibodies are detailed in **Table 4.12.** The IgG sensitised cells were incubated with three replicate slides. To prepare the IgG sensitised cells, group O $R_1r$ cells were selected (presented in **Figure 4.13f)** and these were sensitised with anti-D 'K' (LHM169/80) following the method used for IgG antibody serological testing in Chapter 2. This antibody is of isotype $IgG_3$. Un-sensitised cells (native) were also tested.

**Table 4.12.** Probe antibodies for use in direct antiglobulin testing by microarray.

| Specificity and identity in results | Cell line/ Identity | Antibody Class | Concentration (mg/ml) |
|---|---|---|---|
| Anti-D 'K' | LHM169/80 | $IgG_3$ | 1.09 |
| Monoclonal Anti-$IgG_3$ | LG3A | IgG | 1.20 |
| Rabbit Anti-IgG | Rabbit polyclonal | IgG | 0.39 |
| Monoclonal Anti-$C_3$ | 3G8 | IgG | 1.00 |

The results are presented in **Figure 4.15.** The dark blue bars show how the native cells react with the anti-D 'K', but not with any of the other probes. Once sensitised

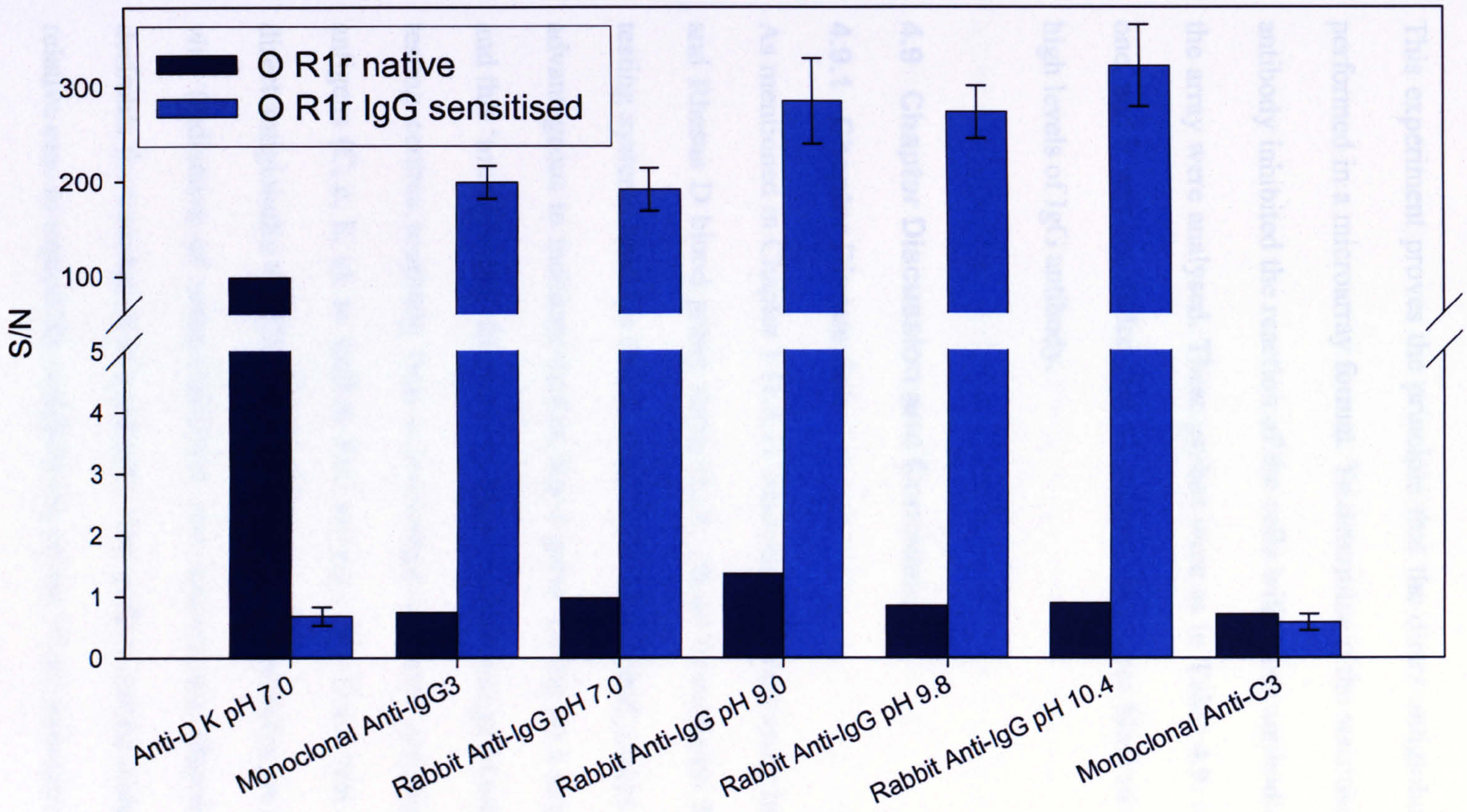



**Figure 4.15**. Results of O $R_1$r erythrocytes both native and IgG sensitised against anti-D, anti-IgG and anti-$C_3$ probes. :
Slide type gold, slide reps 3, pins 700 $\mu$m, probes Table 4.13, probe reps 2, SPM E; blocker PBS-BSA, target/volume: FITC labelled O $R_1$r erythrocyte native/IgG sensitised/450$\mu$l, incubation time 60min, mixing, scanning method B.

with anti-D 'K' (pale blue), the cells do not react with the anti-D 'K' probe, but give very high S/N against the anti-IgG probes. The anti-C3 gives no cross-reactivity.

This experiment proves the principle that the direct antiglobulin test (DAT) can be performed in a microarray format. To determine if the sensitisation by the sensitising antibody inhibited the reaction of the cells with other antibodies, the other probes on the array were analysed. These probes were as in **Table 4.9.** No S/N value was over one, and it was concluded that all antigen sites were blocked due to the presence of high levels of IgG antibody.

## 4.9 Chapter Discussion and Conclusions

### 4.9.1 Chapter Discussion

As mentioned in Chapter 1 (1.3.1), mandatory blood testing includes testing of ABO and Rhesus D blood group status (U.K. Blood Transfusion Services, 2002). Blood testing systems must be able to determine these blood groups. However, it could be advantageous to facilitate further blood group typing on a single microarray format, and this 'multiplexing' feature is an important advantage of microarrays. Many blood testing centres routinely type a percentage of blood donations for further Rhesus antigens (C, c, E, e), as well as Kell antigens (K). Some test centres also perform a direct antiglobulin test (DAT) to determine if erythrocytes are sensitised with IgG *in-vivo* (indicative of some infections and autoimmune disorders, and therefore not desirable for transfusion to a patient). New probes can be added to a microarray with relative ease to exploit the multiplexing power of the microarray format.

However, probes must be well characterised with known and established levels of specificity and activity to meet the industry standards.

A new solid-phase test format must also be able to compete with existing systems in specificity, sensitivity, economy and speed. In terms of sensitivity, weak but clinically significant groups must be detectable without the introduction of false positive reactions. As described in Chapter 1 (1.2.1.1 and 1.2.1.2), rarer blood groups such as $A_x$ and weak D need not be detected in recipients but must be detected in blood donations. Reagents used must not detect the acquired B antigen, which is not a true B antigen and, if detected, can lead to the transfusion of incompatible blood. Scott and Voak (1997) discuss the implications of the use of anti-B reagents that detect acquired B. It is noted that antigenic expression varies between individuals, and that many different examples require evaluation on this system. However, the work presented has shown that ABO, Rhesus, K typing and, in principle, direct antiglobulin testing can be performed using the developed non-agglutination based microarray platform. It was demonstrated that different antibodies were better at the detection of certain antigens e.g. ES9 was good for detection of $A_2$ and ES15 for the detection of $A_x$ and many antibodies require optimisation to improve S/N values.

In this project, the first attempts of blood typing microarrays gave little success. The failure of the early stage approach led to doubt as to whether the antibody–erythrocyte interaction was possible in a microarray solid-phase format. However, there have been several successful attempts of ABO typing in solid-phase systems such as microplates (see Chapter 1, 1.3.5.2.1). It was decided to use microplates as a

solid-phase to evaluate whether another similar solid-phase format might facilitate the reaction. In Chapter 5, the technology of surface plasmon resonance was also used to demonstrate that the solid-phase interaction of probe and erythrocyte could be detected. To develop the blood typing microarray, blood typing was performed in a microplate format. Direct and indirect erythrocyte binding detection methods were both investigated, which do not rely on the occurrence of haemagglutination.

In the blood typing ELISAs performed, it was found that erythrocytes could bind specifically to the antibody coated plates, but that indirect fluorescent labelling methods either prevented binding or caused detachment of the cells. It was apparent that labelling the erythrocytes with fluorescently labelled molecules, such as ConA, inhibited the probe Ab–Ag interaction. It was felt that directly labelling the erythrocyte with a fluorophore, rather than indirectly via another molecule, may improve the retention of the erythrocytes and give a detectable signal. Therefore, direct fluorescent labelling of the erythrocytes was investigated, as it would be a useful tool in the analysis of microarray interactions. The direct labelling concept was used with success in subsequent microarrays. This technique proved very useful in the development stage of the microarrays and led to the successful blood typing of many samples.

Non-agglutination blood typing on a solid-phase has been reported previously by Quinn *et al.* (1997). The authors used the Biacore platform to differentiate between blood types. In this thesis, non-agglutination based blood typing was performed using an open plane, multi-parameter microarray and a fluorescence readout.

However, direct labelling of every blood sample would be impractical for routine high-throughput use, mainly due to timescales, but it would also be an extra step that would require automation. The optimal detection method for high-throughput testing may be a secondary detection molecule added along with other detection molecules. Compatibility with other detection molecules and probes should be considered. This will be of particular importance when amalgamating antibody screening with blood typing, and extending to pathogen testing. This will be discussed further in Chapter 7. For this purpose, other methods to indirectly detect bound erythrocytes were attempted, with some success.

To improve the indirect fluorescent labelling method, the number of fluorophore molecules attached to the detection molecule could possibly be increased. Other detection molecules could also be investigated. The anti-Rh29 binds to an integral membrane protein and it may be more beneficial to select a molecule specific for a peripheral antigen, such as H or glycophorin. If considering a final blood typing microarray system, anti-H is available, but would not detect the rare Bombay phenotype (frequency 1 in 7600 in India, lower elsewhere (review, Daniels, 2002)), which has no H on the cell surface. To address this problem, the anti-H could be used as a probe antibody. In this case, the Bombay phenotype would be demonstrated by the absence of binding to the anti-H control probe. The inclusion of this extra probe would be beneficial, and incremental costs are small. The use of an antibody to glycophorin was used during this development work (data not shown), but gave no meaningful results.

Scanning of unlabelled cells gave an indication that autofluorescence may be a possible form of detection of cell binding if it were optimised. However, the high level of fluorescence seen with directly FITC labelled cells would indicate that the test system might be more sensitive, as many fluorescent molecules may be attached to each cell making it more easily detectable. Nevertheless, autofluorescence may have advantages in certain situations, and this phenomenon was pursued as part of the Proof of Concept project and an Intellectual Property application submitted.

For a probe-ligand interaction to occur, the two active sites must come within close proximity to each other. It is likely that interaction may be increased when movement is facilitated. Because of this, two different incubation methods were investigated. It was found that using Hybridisation Chambers, which allow a larger volume of target solution and mixing, greater S/N ratios were obtained when compared to a smaller incubation volume with minimal mixing. Mixing is difficult when using a small 25 $\mu$l volume, such as that used with Lifter Slips™. A general observation from use of the Hybridisation Chambers is that 'comet tailing' (described in Chapter 3) is less frequent than when using the Lifter Slips™. This may be due to the Lifter Slips™ sliding off the array when washed following incubation, and causing drag of materials over the array. The concentration of erythrocytes in suspension was also investigated to determine if this would affect binding to the array. It was found that a 1 % erythrocyte suspension was optimal, and that more highly concentrated suspensions had a detrimental effect. Incubation time was also investigated as the interaction of solid-phase probe antibodies and targets is different to solution-based agglutination, and required evaluation.

In Chapter 3, polyacrylamide coated slides had shown a superior level of probe protein retention and these, along with poly-L-lysine slides, were selected for investigation in blood typing microarrays. In contrast to the results found in Chapter 3, the poly-L-lysine slides gave far higher S/N levels than the polyacrylamide, when using whole erythrocytes. This is most likely due to the erythrocytes being unable to penetrate the small gaps in the gel (Robb *et al.*, accepted to Transfusion Medicine, 2005, Appendix 4).

Using the optimised procedure, erythrocytes carrying a low number of blood group antigen sites were evaluated on a blood typing microarray. The experiment showed that the microarray blood typing format is capable of detecting clinically significant ABO blood groups. The experiment also demonstrated that the antibodies require optimisation of concentration, especially to detect weak blood groups. In future work, it will be important to use weak antigen cell types to ensure the sensitivity of the antibodies and test system used. This was a crucial development as the potential of the testing platform as a routine test system could be seen. Further development of the system demonstrated it capable of both Rhesus and K typing erythrocytes. The experiments produced some very good results, demonstrating that D, E and c typing can be performed in a microarray format. The only anomaly was the anti-E, which did not detect the $R_2R_2$ cells. It was expected that not all antibodies would show reactivity in solid phase, which was the main reason for using an extensive panel. However, most monoclonal antibodies require extensive testing to determine the optimum reaction conditions (e.g. pH, ionic strength, concentration and possible potentiation), and may or may not benefit from manipulation. The inclusion of higher

avidity reagents may be required to achieve reliable stronger results with weak cell types.

A summary of the observed and expected reactions of the panel of anti-Rhesus antibodies was prepared (**Table 4.13**). In this table, if an antibody has only shown the expected reactivity at one pH, then this was input as the observed reactivity alongside the expected reactivity. Most of the unexpected reactions (shown in white) are unwanted negative reactions, and these antibodies require further development and optimisation. While some probe anti-Rhesus antibodies have shown consistently good results, others have been variable. Although this may be due to optimisation issues, the subject of stability must also be considered. The purification of probe material is important to fabricate spots with specific probes and to minimise NSB. However, it may be that the probe antibodies require the addition of chemical factors to ensure stability. Purification can remove almost all other proteins in the sample as well as salts and sugars, which can provide stability to reagents. Investigation of stability is not covered in this thesis but is considered further in Chapter 7. One antibody that performed well overall (anti-D 'N') was supplied as a freeze-dried material and this may contribute to the overall stability and performance of the antibody. While anti-Ds can vary in their RhD epitope specificity, it is unlikely that this is a factor in the experiments presented as all RhD positive cell samples used were most likely 'normal' RhD positive i.e. positive for all RhD epitopes. Anti-D 'N' is specific for RhD epitope 6/7, as are Anti-D 'D' and 'K', while all other anti-D are specific to a variety of epitopes. In fact, many of these anti-D are part of a kit to determine partial RhD types due to their epitope specificity.

**Table 4.13.** Reactivity of Rhesus antibodies with erythrocytes of different phenotypes. Expressed as observed/expected reactivity. Areas highlighted in green show those giving the desired reactions.

| Ab identity | RBC $R_1R_1$ | RBC $R_2R_2$ | RBC $R_1R_2$ | RBC $R_1r$ | RBC $R_2r$ | RBC rr | RBC r'r' | RBC r''r'' | RBC r''r |
|---|---|---|---|---|---|---|---|---|---|
| Anti-D A | +/+ | +/+ | -/+ | -/+ | -/+ | -/- | -/- | (+)/- | -/- |
| Anti-D B | +/+ | +/+ | -/+ | +/+ | -/+ | -/- | -/- | (+)/- | -/- |
| Anti-D D | -/+ | +/+ | -/+ | -/+ | -/+ | -/- | -/- | (+)/- | -/- |
| Anti-D E | -/+ | +/+ | -/+ | +/+ | +/+ | -/- | -/- | (+)/- | -/- |
| Anti-D F | +/+ | +/+ | -/+ | -/+ | +/+ | -/- | -/- | (+)/- | -/- |
| Anti-D Fsf | -/+ | +/+ | +/+ | -/+ | +/+ | -/- | -/- | -/- | -/- |
| Anti-D G | +/+ | +/+ | +/+ | -/+ | +/+ | -/- | -/- | -/- | -/- |
| Anti-D H | -/+ | +/+ | -/+ | -/+ | -/+ | -/- | -/- | -/- | -/- |
| Anti-D I | -/+ | +/+ | -/+ | -/+ | -/+ | -/- | -/- | (+)/- | -/- |
| Anti-D J | +/+ | +/+ | +/+ | +/+ | +/+ | -/- | -/- | (+)/- | -/- |
| Anti-D K | +/+ | +/+ | +/+ | +/+ | +/+ | -/- | -/- | (+)/- | -/- |
| Anti-D M | -/+ | +/+ | -/+ | -/+ | -/+ | -/- | -/- | -/- | -/- |
| Anti-D N | +/+ | +/+ | +/+ | +/+ | +/+ | -/- | -/- | -/- | -/- |
| Anti-D O | -/+ | -/+ | -/+ | -/+ | -/+ | -/- | -/- | (+)/- | -/- |
| Anti-D P | -/+ | +/+ | -/+ | -/+ | -/+ | -/- | -/- | -/- | -/- |
| Anti-D Q | -/+ | -/+ | -/+ | -/+ | -/+ | -/- | -/- | -/- | -/- |
| Anti-D R | -/+ | -/+ | -/+ | -/+ | -/+ | -/- | -/- | -/- | -/- |
| Anti-D S | -/+ | -/+ | -/+ | -/+ | -/+ | -/- | -/- | -/- | -/- |
| Anti-E | -/- | -/+ | -/+ | -/- | -/+ | -/- | -/- | +/+ | -/+ |
| Anti-c | -/- | +/+ | -/+ | +/+ | -/+ | +/+ | -/- | +/+ | -/+ |

The direct antiglobulin test was performed by microarray in this chapter. The sensitising antibody used was of high potency and therefore, the sensitised cells completely inhibited the reaction with the same probe antibody. The level of IgG sensitisation was perhaps far higher than would be expected in a naturally DAT positive sample, so it is unknown whether this test format would detect real or weakly sensitised samples. It would be expected that a potent antibody might bind to all RhD antigen sites on the cell surface, therefore blocking these sites to any other antibody to the same epitope. Reduced level sensitisation using antibody dilutions would require investigation, to determine if this still affects the typing of other blood groups.

It would be advantageous if the microarray format could correctly group DAT positive samples. In liquid phase testing, the presence of IgG means that all tests give positive reactions when tested with anti-IgG, meaning that IgG blood grouping reagents cannot give accurate results. DAT positive samples are usually tested with IgM typing antibodies. The detection system would be critical when testing DAT positives, especially if blood typing was performed alongside antibody screening. It is likely that the detection system for antibody screening would include anti-human IgG, and this would bind to any DAT positive cells on the array. This would require clarification when comprehensive testing was amalgamated on one microarray.

About midway through this stage of the project, some of the data presented in this thesis, along with the whole concept of blood typing microarrays, was used by the Scottish National Blood Transfusion Service in collaboration with the Scottish

Centre for Genomic Technology and Informatics (University of Edinburgh) to apply for Proof of Concept (PoC) funding from Scottish Enterprise. Based on the work presented in this application, funding was successfully gained and amounted to £200K over a period of two years. This led to the appointment of others to work on the project. With more personnel involved in the work, new developments were made and at times affected the work presented in this thesis. This was mentioned where appropriate. Two major developments were the preparation of new antibody probes by a different method to that used previously, and the use of further slide types for the immobilisation of probes. This input helps demonstrate the huge potential which is recognised in microarray technology.

### 4.9.2 Chapter Conclusions

The work of this chapter has demonstrated the following in response to the chapter aims:

- *Evaluate whether blood typing can be performed in a microarray format, the basis of which was developed in Chapter 3, which will involve the use of erythrocytes of different blood groups, initially Rh and ABO.*

  Using the selected antibody probes, Rhesus D typing was first attempted with no success. This was followed by ABO grouping of both high and low antigen density expression samples performed successfully in a microarray format.

- *Establish detection methods for direct and indirect detection of erythrocytes bound to the blood typing microarray.*

Both direct and indirect methods for the detection of erythrocytes bound to a blood typing microarray were evaluated. Indirect methods using fluorescently labelled lectin were shown to inhibit subsequent blood typing interactions. However, those using direct labelling of the erythrocyte with fluorophore proved highly successful although perhaps impractical for scale-up. Indirect methods using fluorescently labelled secondary reagents were successful, but resulted in lower S/N values which were still suitable for blood typing purposes.

- *Optimise the blood typing microarray procedure to enhance reactions and improve the performance of the test platform.*

  Several parameters, including reaction volume, mixing, incubation time were optimised for successful blood typing using the microarray platform.

- *Once ABO blood typing is achieved and optimised, extend the probe panel to include Rhesus and Kell antibodies to blood group antigens.*

  Rhesus D, E and c typing was performed using a blood typing microarray format. Some probe antibodies gave inconsistent reactions (anti-E and anti-c). Anti-D antibodies showed good correlation with the level of antigen expression. The effect of pH on antibody immobilisation and reactions were investigated and demonstrated individual requirements for different antibodies.

- *Determine the ability of microarray technology to perform detection of direct antiglobulin positive samples.*

Erythrocytes sensitised with IgG antibody were detected by both monoclonal and polyclonal anti-IgG, with no cross reactivity with anti-$C_3$.

In summary, this chapter has demonstrated that ABO, Rhesus D, c and E, K blood typing and direct antiglobulin tests can be performed in a microarray format. The multiplexing ability of the microarray format has been demonstrated, by typing erythrocytes against a multi-parameter panel of immobilised probe antibodies. The potential for comprehensive blood testing by protein microarray technology is clear.

Chapter 5 looks at the use of many of the probe antibodies used in this chapter in a complementary solid-phase system, using surface plasmon resonance.

# CHAPTER 5

# ANTIBODY ANTIGEN INTERACTION ANALYSIS USING SURFACE PLASMON RESONANCE

# 5. ANTIBODY ANTIGEN INTERACTION ANALYSIS USING SURFACE PLASMON RESONANCE

## 5.1 Introduction and Chapter Aims

This chapter utilises the Biacore platform as an alternative solid-phase method for the study of protein-ligand interactions. The principles of surface plasmon resonance (SPR) technology were described in Chapter 1 (1.3.2.5.3). The work detailed herein focuses on the examination of real-time interactions between molecules of interest, and therefore imparts another perspective on these reactions. For work involving SPR, the immobilised substance is referred to as ligand, and the target as analyte.

Before SPR interaction analysis could commence, preparatory work was performed to enable the optimal conditions for immobilisation of ligand to be established and to determine protocols before interaction with analyte. The work of Quinn *et al.* (1997) was used as an initial reference point and procedures were optimised from this.

The performance of probe ligands can be evaluated using SPR, and this knowledge may be used for the selection of reagents. Chapters 3 and 4 have described the use of microarrays for the immobilisation of proteins (mainly antibodies), and an examination of the interactions with target ligands or cells. In most cases the detection of target binding has been by the use of fluorescently labelled reagents. As SPR detects changes in mass on the sensor surface, the use of labelling is not required for detection.

This chapter also demonstrates the use of synthetic carbohydrate probe molecules for capture of specific antibodies. These synthetic antigens are used in two ways; to rank antibodies on performance and to evaluate their potential for use in antibody screening (see Chapter 6).

The aims of this stage of the project were;

- Use SPR as an established solid-phase reference technology and as a control for the new solid-phase microarray technologies presented in earlier chapters.
- Measure the reactivity of antibody immobilised on a solid surface, supplementary to protein microarrays.
- Compare antibodies using SPR and indicate their suitability for use on a solid-phase.
- Compare the interaction of different erythrocyte types and conditions with immobilised antibody.

## 5.2 SPR Process Optimisation

Similar to the preparation of protein microarrays, SPR assays involve the deposition of probe ligands on the surface of the chip before an interaction assay can be performed. However, in contrast to microarrays, SPR chips allow the regeneration and re-use of the surface for further analysis.

There are four main stages involved that are common to SPR assays. These stages are:

**Immobilisation.** A molecule is linked to the sensor chip. For attachment of proteins amine coupling is the immobilisation method used. The attached or coupled molecule is referred to as the ligand. Prior to this stage, optimisation of pH, flow rates, timescales and buffers must be performed.

**Binding.** The target (referred to as analyte) solution is injected to allow interaction with the probe ligand.

**Regeneration.** Bound analyte must be removed from the ligand surface before the chip may be used for another analysis

**Controls.** Controls are included to determine if observed binding is specific by checking for non-specific binding

This section details the procedures that were necessary to optimise and use the chip for interaction analysis. The process flow of SPR experimentation is detailed in **Figure 5.1.** All methods used in this chapter are detailed in Chapter 2.

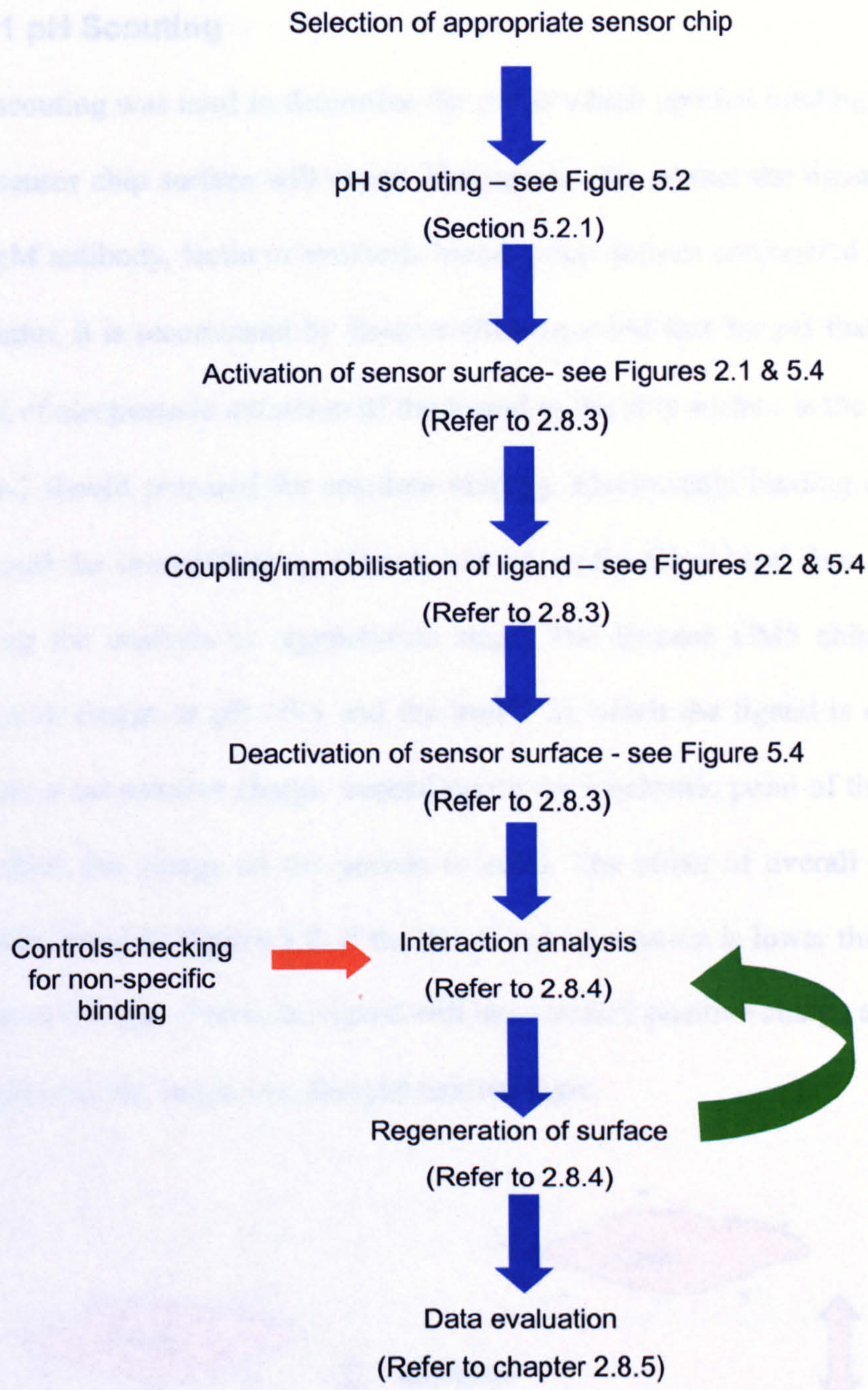



**Figure 5.1.** Surface plasmon resonance process flow diagram. This describes the various stages required to optimise and perform SPR analysis. References are made to the main text.

### 5.2.1 pH Scouting

pH scouting was used to determine the pH at which optimal binding of the ligand to the sensor chip surface will occur. Throughout this project the ligands used are IgG or IgM antibody, lectin or synthetic blood group antigen conjugated to bovine serum albumin. It is recommend by Biacore (Biacore.com) that the pH that has the highest level of electrostatic attraction of the ligand to the chip surface is the pH at which the ligand should prepared for covalent binding. Electrostatic binding of ligand cannot be used for immobilisation since it can be easily dissociated from the sensor chip during the analysis or regeneration steps. The Biacore CM5 chip has an overall negative charge at pH >3.5 and the buffer in which the ligand is delivered should ensure a net positive charge, depending on the isoelectric point of the ligand (the pH at which the charge on the protein is zero). The effect of overall ligand charge is demonstrated in **Figure 5.2.** If the pH of ligand solution is lower than the isoelectric point of the ligand then the ligand will have overall positive charge and, therefore, be attracted to the negatively charged dextran layer.

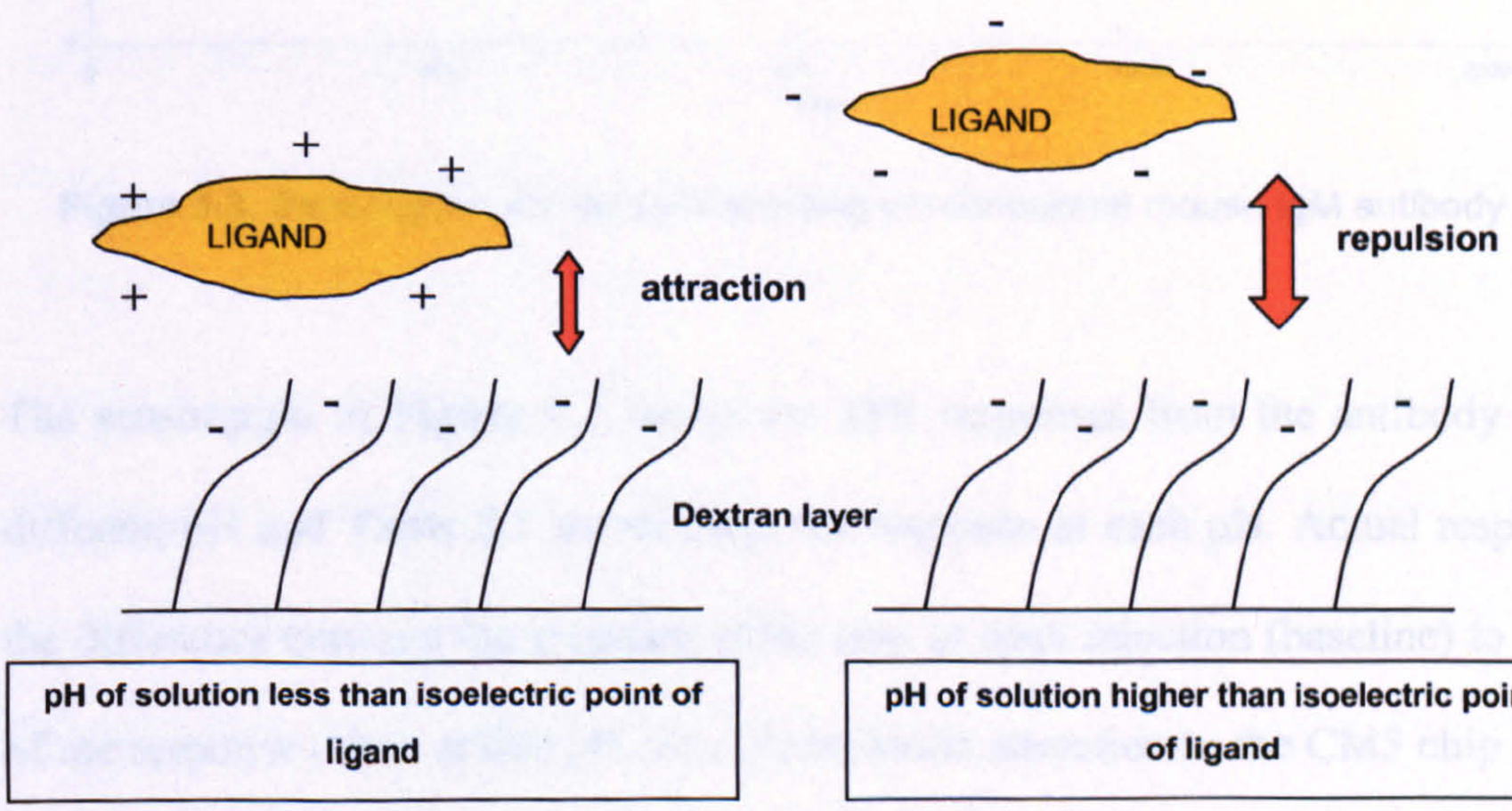



**Figure 5.2.** Schematic of surface plasmon resonance pH scouting principle.

10 mM sodium acetate is recommended as the ligand buffer for most proteins as it has low ionic strength and lacks components that compete with the ligand for active esters on the sensor surface. pH scouting was performed for each ligand to be coupled to a chip, and one example of this process is shown below. The monoclonal mouse IgM anti-A antibody ES9 was used in this example. The concentration of antibody ligand used was approximately 50 $\mu$g/ml. The antibody was dialysed into 10 mM sodium acetate buffer at various pH as indicated in **Figure 5.3**, and then injected over sensor chip surface.

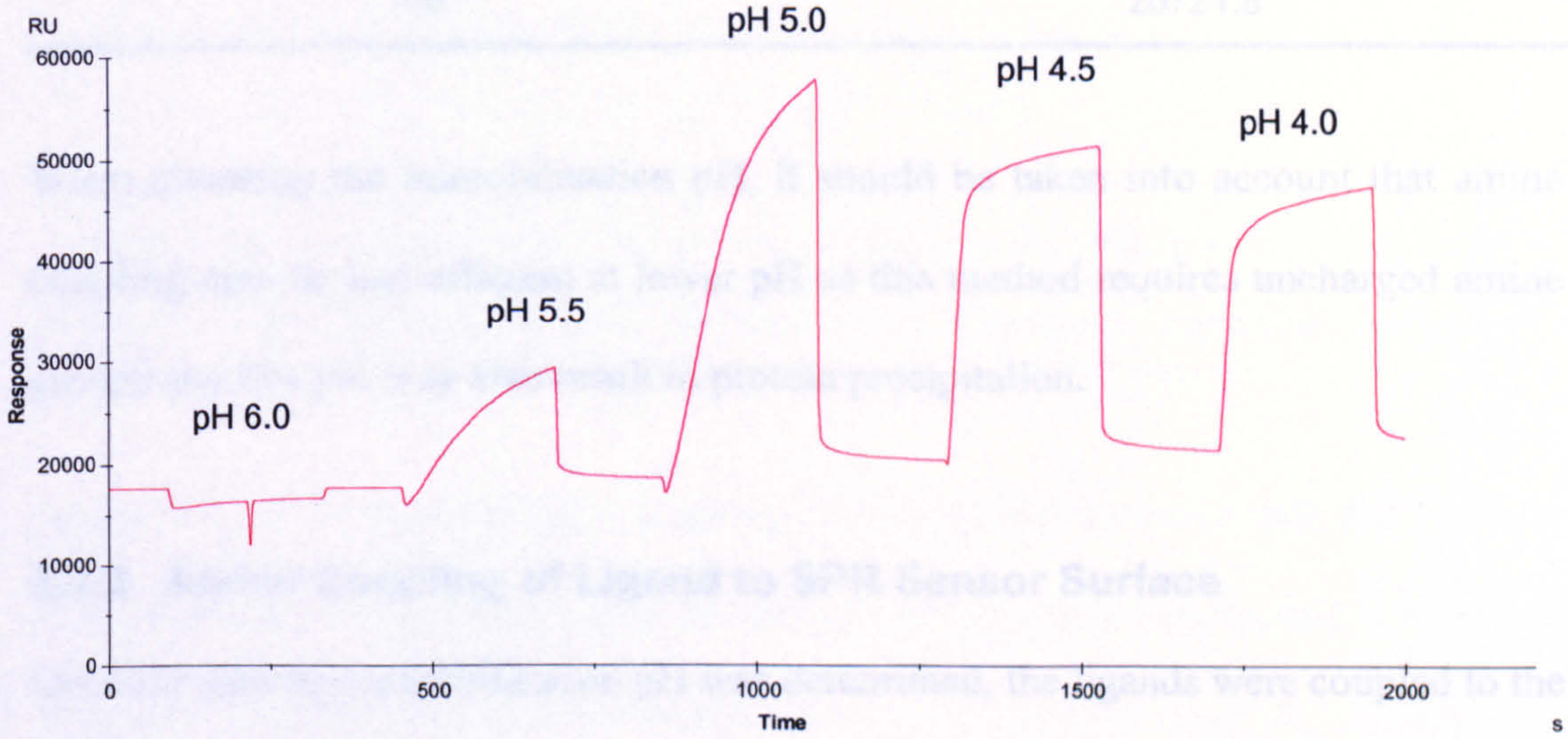



**Figure 5.3.** Sensorgram showing pH scouting of monoclonal mouse IgM antibody ES9

The sensorgram in **Figure 5.3** shows the SPR responses from the antibody ES9 at different pH and **Table 5.1** summarises the response at each pH. Actual response is the difference between the response at the start of each injection (baseline) to the top of the response curve at that pH. The electrostatic attraction to the CM5 chip for this ligand is highest at pH 5.0. This can be determined from both the sensorgram and the

table showing actual response values. A high level of electrostatic binding is also evident at pH 4.5 and 4.0. It is likely that the antibody was more positively charged at the lower pH i.e. at points below the isoelectric point, which will be pH 6.0 or above where no binding is seen.

**Table 5.1**. Summary of pH scouting responses from monoclonal mouse IgM antibody ES9.

| pH of ligand solution | Actual Response (RU) |
|---|---|
| 6.0 | -1188.9 |
| 5.5 | 9084.3 |
| 5.0 | 32527.6 |
| 4.5 | 30676.4 |
| 4.0 | 25721.5 |

When choosing the immobilisation pH, it should be taken into account that amine coupling may be less efficient at lower pH as this method requires uncharged amine groups and low pH may also result in protein precipitation.

### 5.2.2 Amine Coupling of Ligand to SPR Sensor Surface

Once the optimal immobilisation pH was determined, the ligands were coupled to the sensor chip for use in analysis. A description of the coupling process is given in Chapter 2. The Biacore-X™ uses a reference flow cell, a flow cell that has been treated in the same way as the active flow cell, but has had no contact with ligand. The reference flow cell allows the user to perform reference subtraction of binding curves, where the effects of bulk refractive index changes are removed, as well as non-specific binding, from the response in the active flow cell. Reference subtraction can sometimes lead to irregular sensorgrams due to the short time delay in the sample reaching both flow cells. This is depicted in pale blue in **Figure 5.4**, which

shows the 'reference subtraction' response (FC2-1). As any binding of analyte to the reference flow cell is due to non-specific binding, reference subtraction was performed on all data presented in this work.

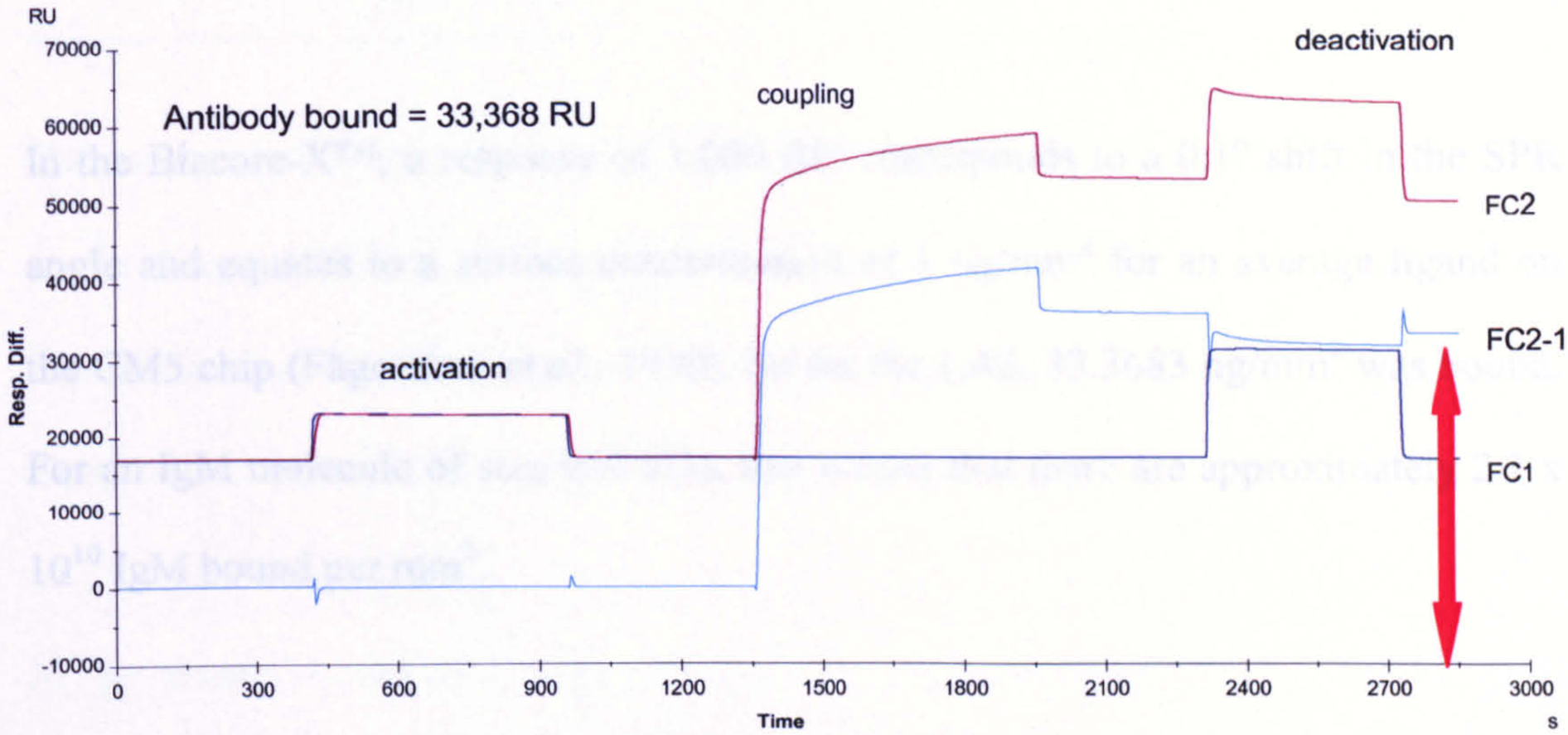



**Figure 5.4**. Sensorgram of amine coupling of IgM antibody, LA2. The level of antibody bound is shown by the red arrow - the response after deactivation, taken from the response in FC2-1, which was 33,368 RU. FC1, FC2 and FC2-1 indicate the responses from flow cell 1, flow cell 2 and flow cell 2 minus 1 respectively.

**Figure 5.4** shows a sensorgram resulting from a typical amine coupling procedure. The mouse monoclonal antibody LA2 was coupled in this example, and it was used later in erythrocyte binding experiments.

### 5.2.3 Immobilisation Level of the SPR Sensor Chip Surface

When coupling a ligand to the SPR sensor chip surface, the potential maximum level of immobilisation can be calculated using the following equation:

Ligand MW/15 = maximum ligand immobilisation RU

e.g. for an IgM antibody molecule: 950kDa/15 = 63,333 RU

For the example in **Figure 5.4** the amount of antibody bound was 33,368.3 RU. It is unlikely that the maximum level of binding would ever be reached due to the size of the IgM molecule, the presence of non-activated esters, or the removal of some ligand at the deactivation stage.

In the Biacore-X™, a response of 1,000 RU corresponds to a 0.1° shift in the SPR angle and equates to a surface concentration of 1 ng/mm$^2$ for an average ligand on the CM5 chip (Fägerstam *et al.,* 1990). So for the LA2, 33.3683 ng/mm$^2$ was bound. For an IgM molecule of size 950 kDa, this means that there are approximately 2.1 x $10^{10}$ IgM bound per mm$^2$.

## 5.3 Effect of Erythrocyte Concentration on Binding Response

As erythrocytes were to be used in analysis, the optimal concentration was determined using ConA as a model ligand. This experiment would also determine if the assay microfluidic system would allow the flow of erythrocytes, and if the ConA could bind them to show a response. As ConA is a lectin with an affinity for mannose groups it is an ideal reagent for use in optimisation experiments involving erythrocytes, as it will bind irrespective of blood group. The optimal concentration for binding to ConA was to be used in subsequent experiment with antibodies. ConA requires the presence of $Mn^{2+}$ and $Ca^{2+}$ ions to bind to mannose, so this was included in the running buffer solution. Erythrocytes were suspended in Biacore running buffer-Mn/Ca, which was used as the running buffer in this experiment. Erythrocyte concentrations and results from this experiment are detailed in **Table 5.2.**

**Table 5.2.** Effect of erythrocyte concentration on binding to coupled ConA.

| Erythrocyte Concentration (x $10^6$ /μL) | Response |
|---|---|
| 0.5 | 21.0 |
| 1.0 | 88.3 |
| 1.5 | 875.4 |
| 2.0 | 721.6 |

The response increases with an increase in erythrocyte concentration. However due to the large difference in binding from 1.0 to 1.5 x $10^6$ erythrocytes/ $\mu$l, this experiment was repeated with a larger range of erythrocyte concentrations, and using immobilised anti-A LA2.

In the repeat experiment, the buffer used was Biacore running buffer, and the cells were of blood group $A_1$. The results are presented in **Table 5.3**, and the effect of concentration demonstrated in **Figure 5.5**. Here, the variability between concentrations is less variable. It was noted that there was a high level of variability between runs, but the mean value was used in the figure. The concentration of 1.76 x $10^6$ erythrocytes/$\mu$l showed the optimal results, although within the range of 1.51-2.4 x $10^6$, results are very similar. Higher concentrations were not analysed due to difficulties in injection over the flowcell caused by packed erythrocytes increasing the viscosity of the solution. All erythrocyte suspensions used in subsequent experiments were at a concentration of 1.76 +/- 0.2 x $10^6$ /$\mu$l.

**Table 5.3**. Effect of group $A_1$ erythrocyte concentration on binding to coupled anti-A LA2.

| Erythrocyte Concentration ($x\ 10^6\ \mu L$) | Response (Run 1) | Response (Run 2) | Mean Response (RU) |
|---|---|---|---|
| 0.95 | 5014.4 | 2442.9 | 3728.7 |
| 1.36 | 5593.4 | 2033.2 | 3813.3 |
| 1.51 | 10301.7 | 13028.7 | 9133.7 |
| 1.76 | 15210 | 5977.3 | 10593.7 |
| 2.05 | 6823.1 | 14724.3 | 9124.1 |
| 2.4 | 14449.8 | 4009.2 | 9229.5 |

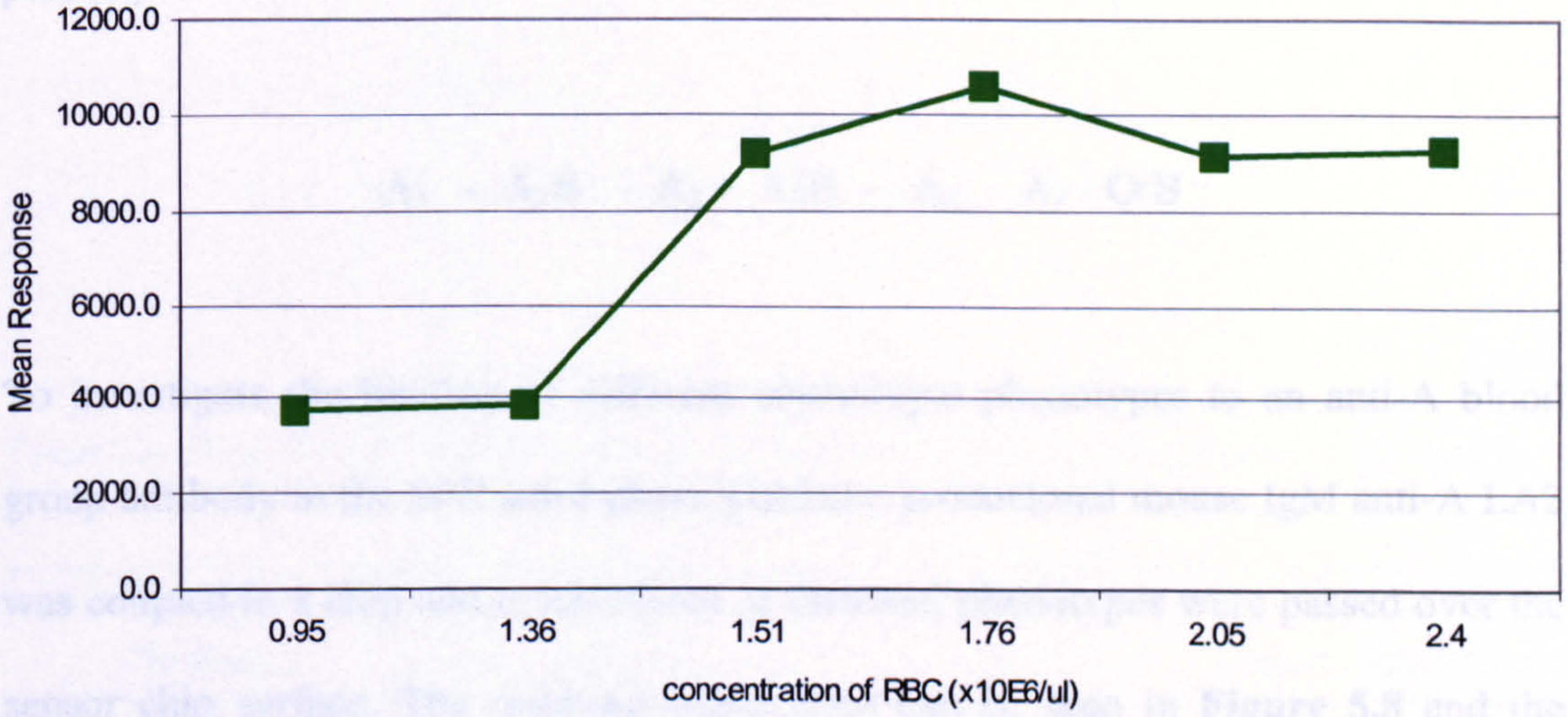



**Figure 5.5**. Line graph showing the effect of erythrocyte concentration on binding to anti-A LA2, using SPR.

## 5.4 Effect of Erythrocyte Antigen Expression Level on Binding to Sensor Chip

In Chapter 4 it was shown that different blood group erythrocytes show varying levels of binding to a blood typing microarray. Different erythrocyte phenotypes have different levels of blood group antigen expression on their surface. This can affect the detection of antigen. **Table 4.6** (Chapter 4) details the average numbers of

blood group A antigen sites per erythrocyte from different phenotypes. If the average number of blood group A antigen sites per $A_1$ erythrocyte is 990,000, then a 50 $\mu$l suspension at 1.5 x $10^6$ erythrocytes/ml will contain approximately 7.425 x $10^{13}$ group A antigen sites.

It would be expected that binding of different blood group erythrocyte types to an anti-A antibody would show a reduction in response as follows (group O and group B erythrocytes are suitable negative controls as there are no group A antigens present):

$$A_1 \text{ - } A_1B \text{ - } A_2 \text{ - } A_2B \text{ - } A_x \text{ - } A_3 \text{ - } O/B$$

To investigate the binding of different erythrocyte phenotypes to an anti-A blood group antibody in the SPR solid-phase platform, monoclonal mouse IgM anti-A LA2 was coupled to a chip and erythrocytes of different phenotypes were passed over the sensor chip surface. The resulting sensorgram can be seen in **Figure 5.8** and the response levels in **Table 5.4**. The experiment was performed twice (runs 1 and 2). The level of repeatability of this analysis is evident when the results are displayed in a line graph **(Figure 5.9)**.

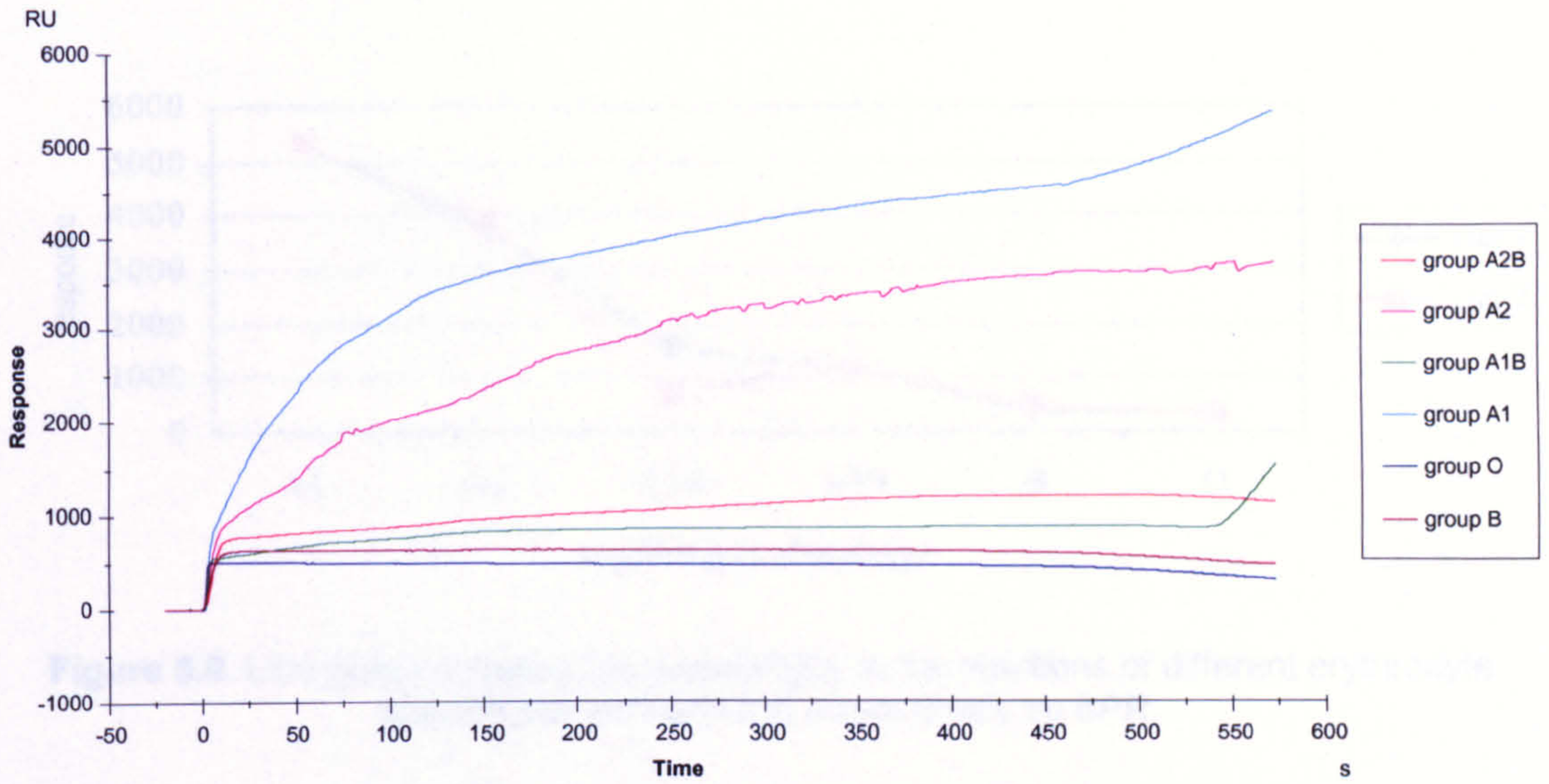



**Figure 5.8.** Sensorgram showing binding of different erythrocyte phenotypes to Anti-A (LA2), run 1 only.

**Table 5.4**. Reactions of different erythrocyte phenotypes with Anti-A (LA2) antibody, results from runs 1 and 2, and serological haemagglutination potency results for comparison.

| Erythrocyte Phenotype | Response (run 1) | Response (run 2) | Potency by haemagglutination |
|---|---|---|---|
| $A_1$ | 5351.5 | 5316.1 | 1024 |
| $A_2$ | 3751.0 | 3874.6 | 512 |
| $A_1B$ | 1558.3 | 702.5 | 512 |
| $A_2B$ | 1126.5 | 1123.9 | 256 |
| B | 384.8 | 452.6 | negative |
| O | 387.0 | 308.5 | negative |

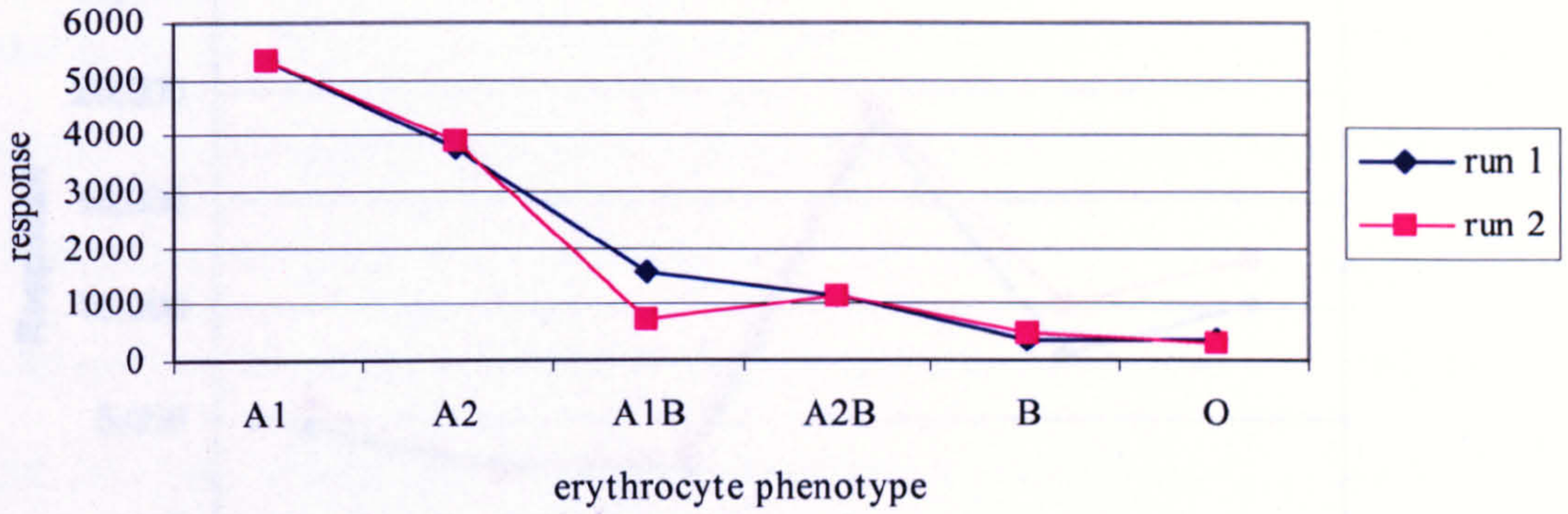



**Figure 5.9**. Line graph showing the repeatability in the reactions of different erythrocyte phenotypes with anti-A (LA2) antibody by SPR.

Variation in the level of binding of different erythrocyte groups is evident and will be discussed at the end of this chapter. This experiment was repeated with further examples of $A_2$ and $A_1B$ cells (identified as X, Y and Z) to determine if the LA2 repeatedly binds $A_2$ better than $A_1B$. The repeat analysis was performed on a new chip over two days. The results are detailed in **Table 5.5**, and the repeatability demonstrated in a line graph, **Figure 5.10**.

**Table 5.5.** Reactions of different erythrocyte phenotypes with Anti-A (LA2), results from repeat experiment.

| Erythrocyte Identification | Response (Day One) | Response (Day Two) |
|---|---|---|
| $A_2$ X | 4,583.2 | 5,785.8 |
| $A_2$ Y | 2,762.2 | 2,525.0 |
| $A_2$ Z | 2,799.3 | 3,503.0 |
| $A_1B$ X | 18,722.8 | 19,292.7 |
| $A_1B$ Y | 7,900.4 | 10,108.4 |
| $A_1B$ Z | 10,191.6 | 12,279.0 |

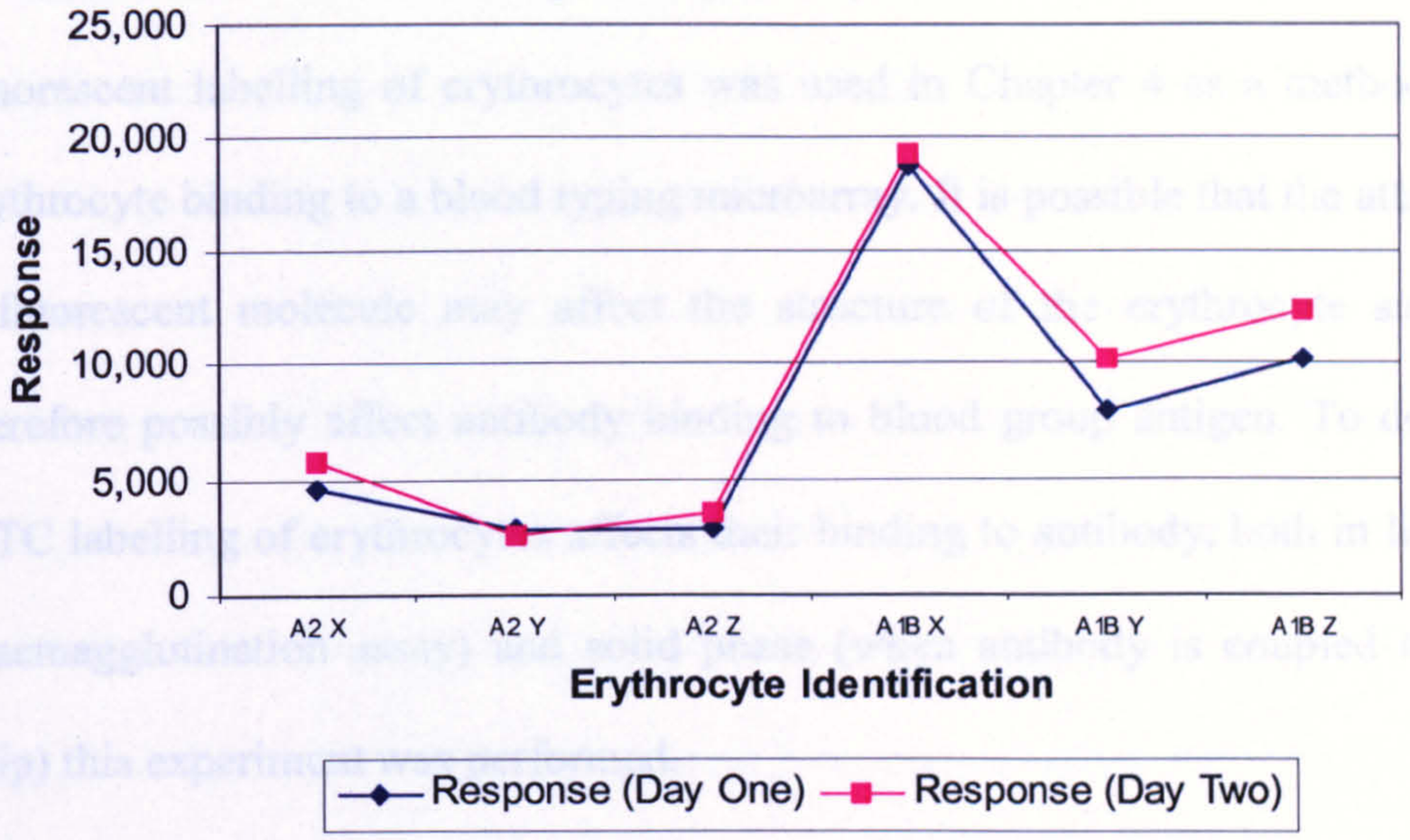



**Figure 5.10**. Reactions of different erythrocyte phenotypes with anti-A (LA2), results from repeat experiment.

On repeat the results are very different to the first experiment. The second experiment was performed with three examples of each type of erythrocyte, from three different donors so is, therefore, a fairer representation of the variety that exists. These results were repeatable over the two days. The haemagglutination results from the repeat experiment are shown in **Table 5.6**, and discussed at the end of this chapter.

**Table 5.6.** Haemagglutination titration assay of $A_2$ and $A_1B$ erythrocytes.

| Erythrocyte | Titration End Point | Titration Score |
|---|---|---|
| $A_2$ (first experiment) | 512 | 109 |
| $A_2$ X | 1024 | 122 |
| $A_2$ Y | 256 | 105 |
| $A_2$ Z | 512 | 107 |
| $A_1B$ (first experiment) | 512 | 107 |
| $A_1B$ X | 2048 | 136 |
| $A_1B$ Y | 1024 | 115 |
| $A_1B$ Z | 1024 | 115 |

## 5.5 Effect of FITC Labelling of Erythrocytes

Fluorescent labelling of erythrocytes was used in Chapter 4 as a method to detect erythrocyte binding to a blood typing microarray. It is possible that the attachment of a fluorescent molecule may affect the structure of the erythrocyte surface, and therefore possibly affect antibody binding to blood group antigen. To determine if FITC labelling of erythrocytes affects their binding to antibody, both in liquid phase (haemagglutination assay) and solid phase (when antibody is coupled to a sensor chip) this experiment was performed.

Erythrocytes of group $A_1$ and B were used. The group B cells were used to determine the effect of labelling on non-specific interactions. An aliquot of the erythrocytes remained unlabelled and another was FITC labelled. All cell samples were prepared to the same concentration as appropriate for the assay, therefore the same number of labelled and unlabelled cells was present in each sample.

For the SPR assay the monoclonal mouse IgM anti-A LA2 was coupled to a chip and the erythrocytes were passed over the sensor chip surface. The potency determination was carried out using haemagglutination assay.

**Table 5.7.** Effect of FITC labelling of erythrocytes on binding to LA2.

| Erythrocyte | Response (run 1) | Response (run 2) | Potency by haemagglutination |
|---|---|---|---|
| $A_1$ unlabelled | 5351.5 | 5316.1 | 1024 |
| $A_1$ FITC labelled | 1214.0 | 667.0 | 1024 |
| B unlabelled | 384.8 | 452.6 | 0 |
| B FITC labelled | 35.5 | 123.5 | 0 |

This shows that the FITC labelling results in an average of 82.37% reduction in specific binding to the LA2, and an 81.01% reduction in non-specific binding in the SPR assay. This is a large amount, and from this it was deduced that the labelling procedure was affecting the antibody-antigen reaction on this analysis platform. However, as the FITC labelling did not appear to restrict binding of cells to the blood typing microarray, this was later repeated using more examples of labelled and unlabelled cells. **Table 5.8** details the results from the repeat experiment using two examples of group $A_1$ cells and a group B negative control. All conditions were the same as before.

**Table 5.8**. Effect of FITC labelling of erythrocytes on binding to LA2, repeated.

| Erythrocyte | Response (run 1) | Response (run 2) | Mean response (RU) |
|---|---|---|---|
| $A_1$ unlabelled (1) | 10301.7 | 13028.7 | 9133.7 |
| $A_1$ FITC labelled (1) | 11261.2 | 5912.2 | 6376.0 |
| $A_1$ unlabelled (2) | 8772 | 15268.2 | 12020.1 |
| $A_1$ FITC labelled (2) | 15485.1 | 8728.4 | 12106.8 |
| B unlabelled | -10.1 | 16 | 3.0 |

In the repeat experiment, the FITC labelling did not appear to affect the binding of erythrocytes to the chip to the same level as in **Table 5.8.**

## 5.6 Synthetic Antigen Binding

Isolation of blood group antigens from the erythrocyte membrane can be problematic, especially when trying to retain the antigen in its functional state. Isolated and functional blood group antigen could potentially be used to evaluate the binding of different blood group antibodies, or could be used in the antibody screening of human serum samples. The blood group A and B antigenic determinants

are carbohydrate and can therefore be synthesised and conjugated to BSA. To determine if an immobilised antibody would specifically bind to synthetic carbohydrate antigen, an interaction analysis was performed using synthetic A and B antigens. The resulting sensorgram can be seen in **Figure 5.11.** The response from bound synthetic antigen is presented in **Table 5.9**.

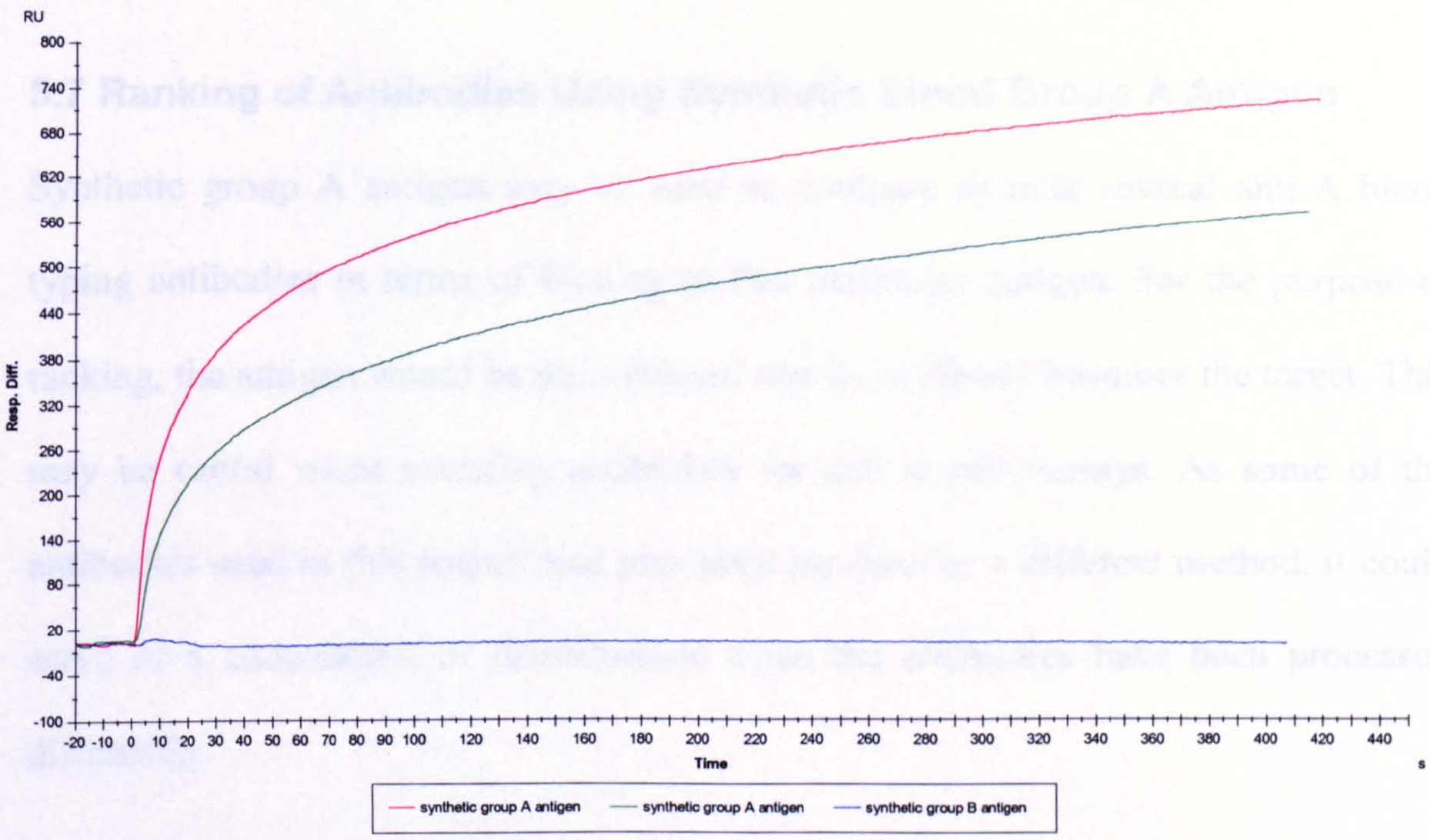



**Figure 5.11.** Sensorgram of synthetic group A and B antigen binding to Anti-A LA2.

**Table 5.9**. Synthetic A and B antigen binding to Anti-A LA2 on an SPR chip.

| Synthetic Antigen | Response (first) | Response (repeat) |
|---|---|---|
| A | 736.9 | 615.0 |
| B | -43.0 | 0.7 |

The results show that the LA2 antibody specifically binds the synthetic group A antigen. Therefore, it was concluded from this experiment that the synthetic antigens could be useful in an experiment to 'rank' the blood typing antibodies that are available and determine which may be suited for use in another solid phase system for blood typing. Also, the synthetic antigens were to be investigated for suitability for use in microarray-based blood group antibody screening methods (Chapter 6).

## 5.7 Ranking of Antibodies Using Synthetic Blood Group A Antigen

Synthetic group A antigen may be used to compare or rank several anti-A blood typing antibodies in terms of binding to that particular antigen. For the purpose of ranking, the antigen would be immobilised and the antibody becomes the target. This may be useful when selecting antibodies for use in microarrays. As some of the antibodies used in this project had also been purified by a different method, it could serve as a comparison of functionality when the antibodies have been processed differently.

Synthetic blood group A antigen conjugated to BSA was coupled to a CM5 chip. A selection of available IgM anti-A blood group antibodies was prepared to a concentration of 50 $\mu$g/mL, before injection over the sensor chip surface. The antibodies had been purified by different methods; gel filtration denoted as (*) and by affinity column denoted as (**). The responses are presented in **Table 5.10**, and the sensorgram as **Figure 5.12.**

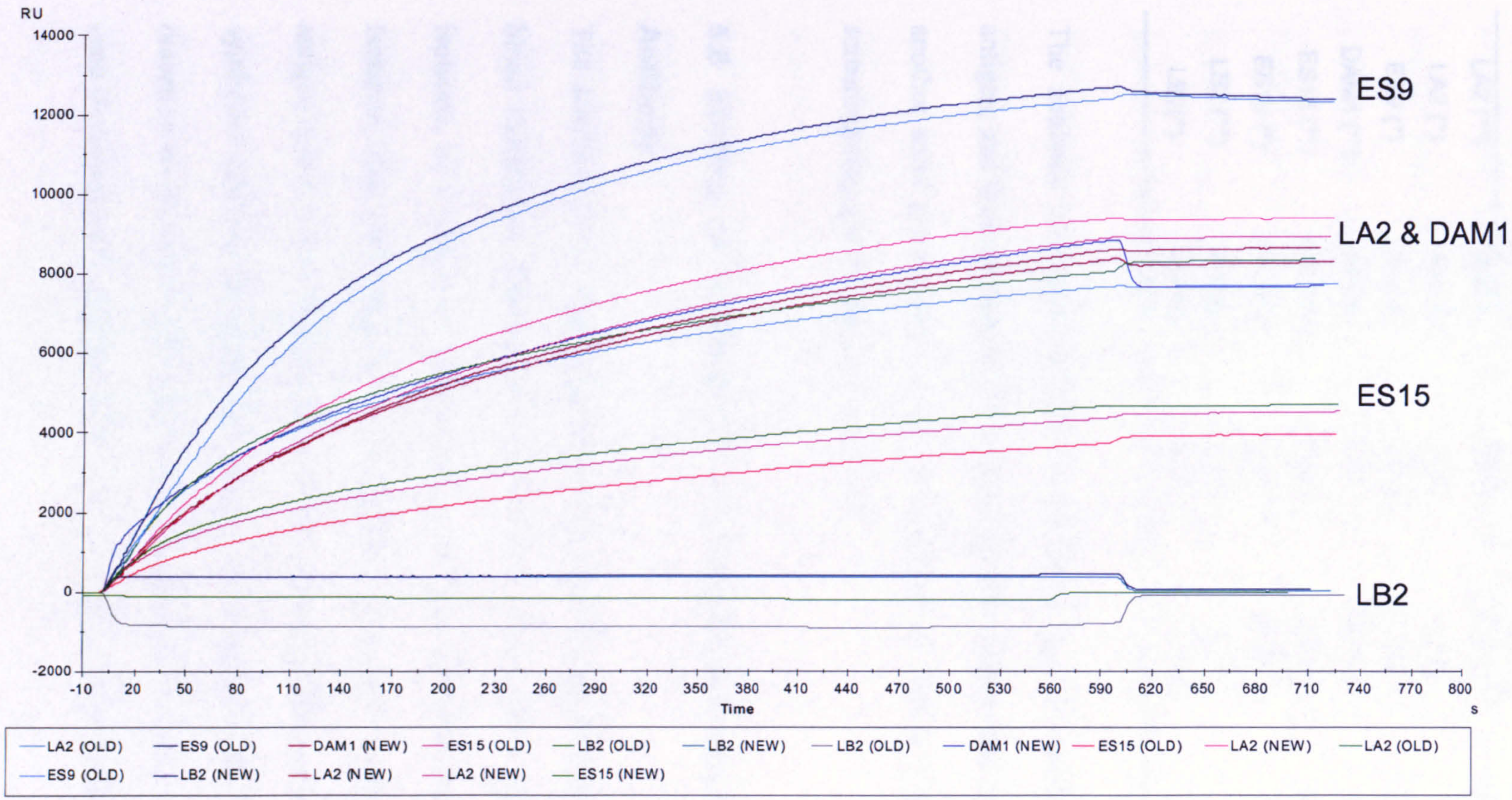



**Figure 5.12**. Sensorgram showing ranking of antibodies against synthetic blood group A antigen. In this figure 'old' refers to gel filtration (*/OLD) purification and 'new' to affinity purified (**/NEW).

**Table 5.10.** Responses in ranking of antibodies against synthetic group A antigen.

| Antibody Identification | Antibody Specificity | Response (first) | Response (repeat) | Mean Response | Rank |
|---|---|---|---|---|---|
| LA2 (**) | Anti-A | 9267.8 | 8431.1 | 8849.45 | 2 |
| LA2 (*) | Anti-A | 8114.3 | 7573.9 | 7844.1 | 4 |
| ES9 (*) | Anti-A | 12393.5 | 12503.1 | 12448.3 | 1 |
| DAM1 (**) | Anti-A | 8112.7 | 7816.3 | 7964.5 | 3 |
| ES15 (**) | Anti-A(B) | 4605.1 | 4158.6 | 4381.7 | 5 |
| ES15 (*) | Anti-A(B) | 4369.0 | 3795.6 | 4082.3 | 6 |
| LB2 (**) | Anti-B | 83.1 | 39.7 | 61.4 | 7 |
| LB2(*) | Anti-B | -103.5 | -177.8 | -140.65 | 8 |

The antibody ES9 gives the best response against the synthetic blood group A antigen, and therefore would theoretically perform better than the other antibodies in another solid phase system such as microarrays. This is a potential method for screening reagents for use in microarrays.

## 5.8 Binding of Erythrocytes to Immobilised Anti-D Blood Typing Antibody

The detection of the RhD antigen on donor and patient erythrocytes is vital for safe blood transfusion. The structure and location of the RhD antigen means that the isolation of a structural and functional antigen for evaluation is very difficult. Isolation may result in a linear polypeptide chain that is unrepresentative of the antigen epitopes and therefore is unlikely to be recognised by anti-D. It is possible to synthesise peptides that represent RhD antigen epitopes but these are outwith the detection levels of the SPR system (approximately 1,500 Da), but could possibly be used if conjugated to a larger molecule for immobilisation e.g. BSA.

Blood typing microarrays using anti-D of both immunoglobulin class IgG and IgM had been attempted in Chapter 4, but were unsuccessful in the early stage development. The interaction analysis provided by SPR was used to determine if specific binding could be detected. Experiments included immobilisation of both IgG (ESD1) and IgM (LDM3) purified antibodies but no binding was detected. Erythrocytes of different Rhesus phenotypes were used in the study. Those of types $R_1R_1$, $R_1r$ and rr were selected and the number of RhD antigens present on each is on average 22,900, 14,600 and zero respectively (reviewed in Daniels, 2002). No binding was evident with any sample during SPR analysis. Papainised erythrocytes were also passed over the flow cell and again no reactions were detected.

All antibody ligands used in this experiment were tested in liquid phase haemagglutination techniques and found to react strongly with the RhD positive cells used in the SPR assay. During the development of the blood typing microarray in Chapter 4, several anti-D were found to react with RhD positive erythrocytes only once procedures had been optimised. This subject is discussed in more detail at the end of this chapter.

## 5.9 Chapter Discussion and Conclusions

### 5.9.1 Chapter Discussion

The Biacore-X™ is a useful tool for the real-time analysis of antibody-antigen interactions, although there are other SPR systems available (Gizeli & Lowe, 1996). Biacore SPR gives reproducibility and sensitivity in experiments. However, one major disadvantage of the Biacore-X™ is the low throughput.

Initially in this chapter, the Biacore platform was optimised for use in the subsequent experiments. The first step was to determine the optimal pH of each ligand prior to immobilisation. However, the pH scouting exercise is only an indication of the optimal binding pH. The accurate optimal binding pH is determined by the amine coupling procedure. Using amine coupling for this purpose would mean the use of multiple chips and large quantities of ligand, which is impractical. Following recommendations by an expert in the field (Dr. P. Brown, Medical Research Council), the pH identified as optimal during pH scouting was used in the coupling. Another consideration is the possible change to the ligand function when immobilised at low pH. The buffer used during interaction analysis is optimal for the interactions and therefore, the specific binding of ligand would not be expected to change, unless it has been denatured by the low pH.

Following pH scouting, immobilisation procedures by amine coupling were evaluated and suitable conditions established for the procedure. IgM and IgG antibodies, lectin and synthetic blood group antigen were all coupled for use in SPR analysis. The maximum immobilisation for each ligand was calculated, and routinely the actual level immobilised was around 50 % of the calculated maximum. The use of amine coupling means that ligands are attached via free amino groups on the molecule. An IgM antibody is a large molecule (approximately 950 kDa) that may bind more than once to the sensor chip surface. The antigen binding sites of antibody molecules could be coupled to the sensor chip surface, making the antibody/antigen reaction more unlikely. Therefore, using amine coupling with antibody molecules means that the orientation of the antibody is random.

The analyte binding capacity of the chip surface is highly dependent on the quantity of ligand bound, as well as the integrity and activity of the ligand. At saturation (when all activated sites are coupled with ligand) this is known as the maximum response ($R_{max}$) and can be calculated as follows:

$(R_{max})$ = analyte MW/ ligand MW x immobilised amount x stoichiometric ratio

For example, the $R_{max}$ if using synthetic group A antigen as analyte and monoclonal mouse anti-A (LA2) as ligand, may be calculated as follows:

82,563/950,000 x 33,368.3 x 10 = 28,999.863 maximum response

This experiment was performed in 5.6, where the response found was only 2.5 % of this $R_{max}$, demonstrating that the theoretical value is not met. This may be due to the presence of non-activated sites, inactive ligand/analyte, or removal of ligand at deactivation stage. It may be possible to increase the response by activating the surface for longer time periods or running the interaction analysis for longer. However, when using relatively large molecules as analyte the importance of saturation of the surface is debatable. For example, an erythrocyte (7.8–8.3 $\mu$m in diameter) may give the same response on binding to one IgM molecule as it would if it were to bind to 10 due to saturation of the surface with the large erythrocytes. The actual value obtained may be sufficient to perform analysis. Therefore, saturation of all possible ligand immobilisation sites may not be required to produce a measurable response.

Following process optimisation procedures, erythrocytes were used in the Biacore. Using immobilised ConA and anti-A LA2, the optimal erythrocyte concentration for use in SPR was determined. This work also allowed the optimisation of conditions for erythrocyte-antibody analysis. The SPR platform was used to analyse interactions between antibody and erythrocytes. It was found that the binding response of different blood group erythrocytes to immobilised antibody varied depending on blood group and antigen expression. This phenomenon is expected and has been reported in other solid-phase methods such as those on microplates (Scott, 1991) and was observed in experiments in Chapter 4 **(Figure 4.14)**.

$A_2$ erythrocytes bound to anti-A LA2 to a higher level than $A_1B$. This may be indicative of the fit of this antibody-antigen interaction i.e. the LA2 antibody may find the group A antigen site less accessible if the B antigen is present. It may be that the group $A_2$ cells selected had an exceptionally high level of antigen expression, or that the $A_1B$ sample selected had less $A_1$ antigen than average i.e. much of the available H sites (refer to Chapter 1, 1.2.1.1) have been converted to group B rather than $A_1$. This cell line has always shown similar reactivity with $A_1$, $A_2$ and $A_1B$ erythrocytes when tested in haemagglutination assays (personal communication, Professor R. Fraser, SNBTS R&D). However, in this SPR assay it showed a marked difference in reactivity between $A_2$ and $A_1B$.

This experiment was repeated with blood samples from three different donors. It could be assumed that the $A_2$ erythrocytes used in the first experiment were unusual in their expression of blood group antigen A i.e. they had an unusually high level of

A antigen for a group $A_2$. To investigate this, potency determination by haemagglutination assay was performed **(Table 5.7)**. From the serological haemagglutination assay carried out it appears more likely that the $A_1B$ had a lower than average level of antigen, therefore it reacted similarly to the $A_2$.

Although the levels of binding were marginally lower in the second round of the experiments the trend was the same. The drop in response levels may be due to some loss of ligand during the regeneration phases, or from the incomplete removal of erythrocytes, or erythrocyte fragments. Data from this experiment demonstrated that SPR offers a repeatable test platform for antibody-erythrocyte assay, and that the response is sensitive enough to demonstrate antigen level variation. This also confirms the trend found when using the blood typing microarray as another solid-phase format. When reactions of the probe antibody LA2 were compared between SPR and microarray platforms, the results both demonstrated the same drop in response/reactivity as was expected from a reduction in antigen site density of the erythrocytes used (i.e. $A_1$ to $A_1B$ to $A_2$).

Analysis of FITC labelled cells showed that the labelling procedure was affecting the antibody-antigen reaction on this analysis platform, and indicated that perhaps the erythrocytes should be unlabelled for optimal detection. Due to this discovery and the fact that FITC labelling is unlikely to be compatible with routine blood testing, methods for the detection of unlabelled erythrocytes were investigated (detailed in Chapter 4). As FITC labelling did not appear to affect erythrocyte binding to a blood typing microarray the experiment was repeated. On repeat the FITC labelling did not

affect the binding of the cells to the sensor chip. This inconsistency may have been due to age of the cells at the time of labelling, those that are older being more affected by the FITC treatment involving increase of the pH of the sample. Due to the nature of these results, it was concluded that it would be worthwhile investigating alternative forms of detection.

Using immobilised anti-A LA2, specific binding of synthetic blood group A antigen was demonstrated. Due to this result, it was decided to use this antibody-antigen pairing in reverse, where the synthetic antigen was immobilised and used to analyse antibody. This was used to compare, or rank, several blood group antibodies in an effort to determine suitability for use in solid-phase testing. The SPR analysis showed that the antibodies performed in the order ES9-LA2/DAM1-ES15 where ES9 was the best reactor. Data from blood typing microarrays has shown that the antibodies ES9 and LA2 perform very similarly. **Figure 4.14** (Chapter 4) showed the binding of different blood group erythrocytes to these antibodies at similar concentration (ES9 at 40.5 and LA2 at 55.0 $\mu$g/ml). In microarrays against group $A_1B$ cells, the ES9 performed better than the LA2, but against $A_2$ and $A_x$ cells they were very similar. When looking only at reactions with cells of group $A_1B$, the reactions followed that described in the SPR experiments i.e. ES9 was best followed by LA2 and ES15. The experiment also showed that the antibodies purified by the different methods gave very similar results when tested, and the high level of repeatability of SPR. The method developed here could potentially be used to screen potential antibody probes and be useful for their selection for use in solid-phase systems, such as microarrays.

In the early stage development, anti-D blood typing by microarray had shown no success. SPR was used as an alternative solid-phase system to determine if the anti-D antibodies would bind RhD positive erythrocytes. Erythrocytes of varying RhD antigen expression were used and no SPR response was recorded.

Enzymes such as papain are often used in blood typing serological methods to enhance the agglutination reactions between certain antibodies and antigens. Papain treatment of erythrocytes removes some blood group antigens from the surface of the cell and lowers the sialic acid level, and therefore the overall charge on the cell. This can enhance the antibody and antigen interaction since they are more accessible to each other, and a reaction may take place which otherwise may not. The use of papainised cells is known to enhance detection of anti-Rhesus antibodies, but did not give a binding response in SPR analysis.

It has been reported (John Allan, Director Alba Bioscience, personal communication) that the presence of surfactant agents such as Tween can affect the antigen binding capacity of some anti-D reagents. The level of surfactant in the running buffer used in the SPR is necessary for injection of the viscous erythrocyte suspension, and is also at such a low level that it is unlikely to interfere with an antibody-antigen interaction.

Rhesus grouping by microarray became successful during the development of the blood typing microarray (Chapter 4), where reactivity of a large panel of anti-D antibodies was evaluated. Anti-D which showed microarray reactivity included

ESD1 (IgG) but not LDM3, in fact no IgM anti-D was reactive. Therefore, it was concluded that the use of a solid phase format for attachment of anti-D antibodies must somehow interfere with the function of some anti-D antibodies. As they are all reactive in liquid phase, it may be that the attachment restricts conformational change of the antibody and does not allow the antigen-binding site to manoeuvre to fit the antigen. The problem may lie with the accessibility of the antigen site on the erythrocyte. As the antigens are integral to the membrane the antibody may not come within the proximity required to interact with the antigen and the erythrocytes bounce off the surface of the chip due to the flow. The flow may cause reduced interaction, as cells only cross the surface in one direction and are not mixed like in microarray.

SPR technology can use a variety of immobilisation methods to capture ligand on the sensor surface. Given the problems associated with RhD antigens, especially their requirement to be inserted into the membrane, then the technology of liposomes could be used to immobilise membrane proteins. Liposomes are fluid filled pouches made up of phospholipids resembling cell membranes and work by accommodating proteins and allowing them to take on their natural state. If isolated RhD proteins were available this may be one system that could be used to study the interactions with antibodies. Another option is the use of peptides that represent the antigen epitopes. However, the peptides may require conjugation to larger molecules for immobilisation due to their size, which could be below the detection limits of SPR. Were either of these methods successful, they could potentially be used in a

microarray system for antibody screening purposes. These are areas of further research identified from this work.

This SPR work has provided much useful information that can be applied when using the same reagents in blood typing microarrays. It has provided a more detailed interaction analysis than can be obtained in microarray. The use of microarray gives a yes or no answer, which is what is required in diagnostics. SPR has demonstrated the effect of solid phase immobilisation on the reactivity of antibodies, the effect of fluorescent labelling and concentration of erythrocytes, has differentiated the reactivity of several antibodies of same specificity, and has shown the ability of antibodies to bind erythrocytes expressing different levels of antigen.

It is doubtful whether this platform could be suitable for high throughput testing which is required for routine blood donation diagnostics. In Scotland alone, over 1000 blood donations are tested every 24 hours. Sensor chips would have to be developed onto which many different ligands could be immobilised and then the technology to be able to evaluate the reactions of one analyte sample with every ligand. The system would also have cost implications as it would possibly be prohibitively expensive. Any new method for blood donation diagnostics would have to compete with the existing costs and timescales of current testing. The fact that the SPR response does not differentiate between specific binding of analyte and non-specific binding from the analyte solution also questions the systems use in medical diagnostics. In this study washed erythrocytes were used. The use of whole blood,

which may be required for full donation testing, may lead to high levels of non-specific binding caused by high protein levels.

### 5.9.2 Chapter Conclusion

- *Use SPR as an established solid-phase reference technology and as a control for the new solid-phase microarray technologies presented in earlier chapters.*

  SPR was used as a long established platform for the study of protein interactions, and therefore enabled a comparison with data gained in microarrays. The ABO blood group antibodies all reacted with antigen using SPR and microarray, but Rhesus D typing was not facilitated by the SPR format.

- *Measure the reactivity of antibody immobilised on a solid surface, supplementary to protein microarrays.*

  SPR in the form of Biacore has been used to demonstrate specific binding of erythrocytes to immobilised antibodies, and thus has confirmed the findings of the blood typing microarray work carried out in Chapter 4. This stage has shown that immobilised antibodies are reactive in solid-phase and can show differential binding of different blood group antigens on erythrocytes.

- *Compare antibodies using SPR and indicate their suitability for use on a solid-phase.*

  Using a reverse arrangement with immobilised antigen (synthetic), the SPR system was used to rank antibodies to allow an insight into which perform well in a solid-phase system.

- *Compare the interaction of different erythrocyte types and conditions with immobilised antibody.*

  As SPR does not rely on labelling of analyte for detection, and therefore allowed a study of the effect of erythrocyte binding to the array when fluorescently labelled and unlabelled.

# CHAPTER 6

# BLOOD GROUP
# ANTIBODY SCREENING BY MICROARRAY

# 6. BLOOD GROUP ANTIBODY SCREENING BY MICROARRAY

## 6.1 Introduction and Chapter Aims

As described in Chapter 1 (1.3.1), erythrocyte antibody screening and reverse antibody screening are mandatory components of blood donation testing. This chapter looks at the development of a novel microarray-based technique for blood group antibody screening, which involves the immobilisation of antigens for detection of antibodies.

The result of reverse antibody screening is used to confirm the forward ABO blood typing performed. To perform reverse ABO grouping of donors and patients, the current guidelines (U.K. Blood Transfusion Services, 2002) state that, as a minimum, plasma/serum should be tested against cells of type $A_1$, B and O. Cells used for this purpose may be pooled donations from individuals of identical ABO group.

Guidelines for testing of blood donors and recipients varies with respect to erythrocyte alloantibody screening, and the minimum blood group antigens required for each use are:

Donor testing: D, C, c, E, e, K

Patient testing: D, C, c, E, e, K, k, $Fy^a$, $Fy^b$, $Jk^a$, $Jk^b$, S, s, M, N, $P_1$, $Le^a$, $Le^b$

(For full characterisation of antigens see **Table 1.4**).

Cells for patient testing must be from a minimum of two individuals, should not be pooled and at least one cell phenotype should be Rhesus $R_2R_2$ and another $R_1R_1$

(Guidelines for the Blood Transfusion Services in the United Kingdom, U.K. Blood Transfusion Services, 2002) (for descriptions of Rhesus phenotypes see **Table 4.10**). Homozygous $Fy^a$, $Fy^b$, $Jk^a$, $Jk^b$, S, and s expression of antigens is also recommended. All reagent erythrocytes for this purpose must be group O, should not express blood group antigens present in less than 1 % of the population, and should not be used if shown to cause reactions with Human Leucocyte Antigen (HLA) specific antibodies. HLA antigens, if present, can cause unwanted clinically insignificant reactions with certain HLA antibodies.

The principles of solid-phase testing are outlined in **Figure 6.1**. Existing solid-phase methods use either immobilised erythrocyte membranes or anti-human globulin reagents. Erythrocyte antibody screening by microarray has not been described yet in the available literature.

For comprehensive blood testing, the inclusion of an antibody screen is mandatory. As the use of the microarray format for blood typing has already been demonstrated in this thesis, this chapter will look at the use of this format for erythrocyte antibody screening. For this purpose, different forms of antigen presentation will be used: whole erythrocytes which retain the antigens in their native environment, erythrocyte membrane (protein) fragments which contain integral and membrane-associated proteins in a non-denatured state based on their association with cellular membranes, and synthetic carbohydrate antigens conjugated to BSA.

Therefore, the aims of this final stage of the project were:

- Investigation of different slide surface chemistries for the immobilisation of novel blood group antigens.
- Evaluation of blood group antigens in a variety of forms for their use in antibody screening – whole erythrocytes, erythrocyte membrane (protein) fragments and synthetic antigens.

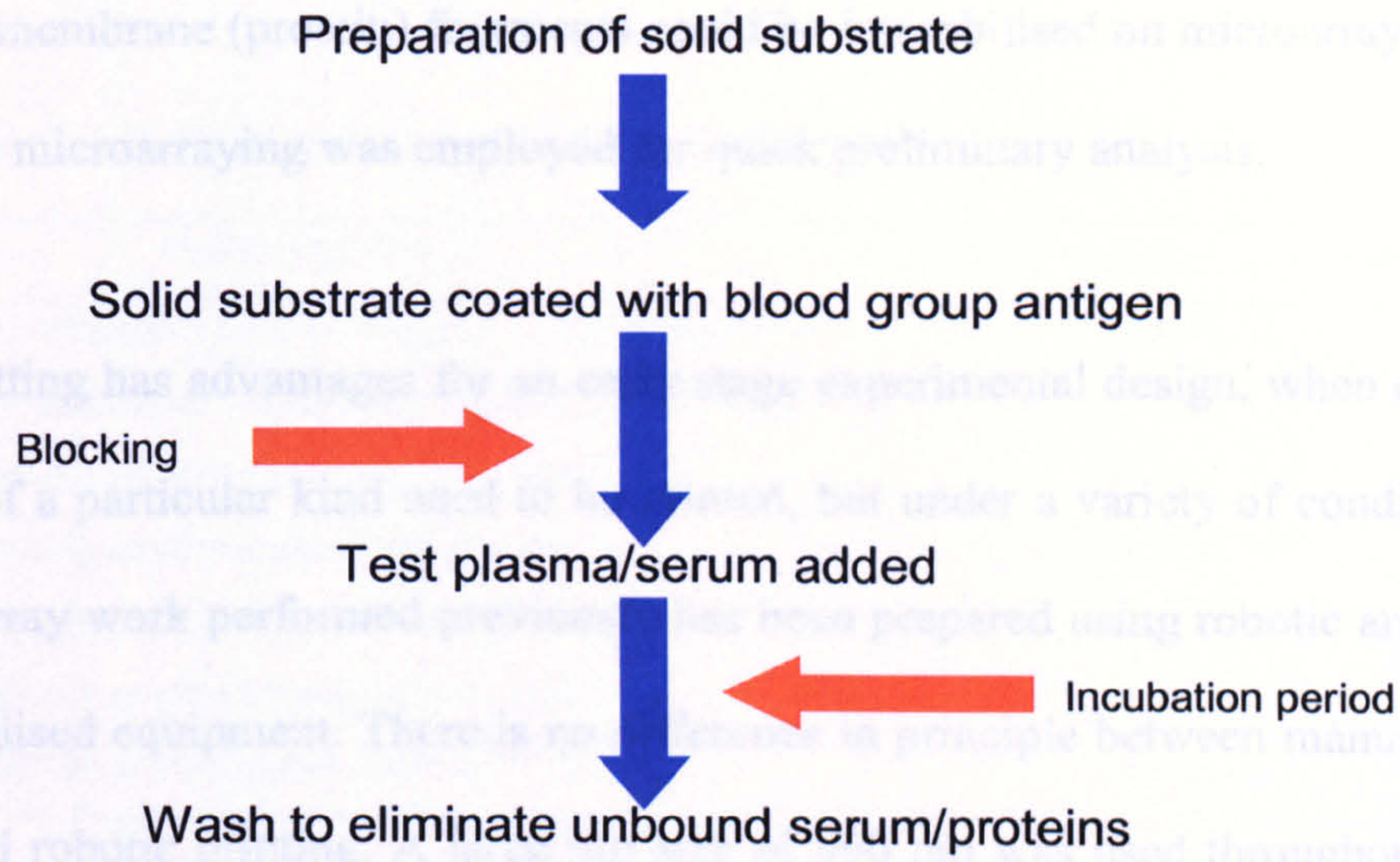



**Figure 6.1.** Basic principles of solid phase antibody screening.

Details of all methods and reagents used can be found in Chapter 2. The information that defines the protein microarray printing, processing characteristics and target

solutions for each experiment will be detailed in the figure legends, and as used in Chapters 3 and 4.

## 6.2 Evaluation of Erythrocytes and Membrane Protein Fragments for Antibody Screening

This experiment was performed to determine whether whole erythrocytes and erythrocyte membrane (protein) fragments could be immobilised on microarrays. For this, manual microarraying was employed for quick preliminary analysis.

Manual spotting has advantages for an early stage experimental design, when only a few slides of a particular kind need to be printed, but under a variety of conditions. The microarray work performed previously has been prepared using robotic arrayers and standardised equipment. There is no difference in principle between manual and programmed robotic printing. A large pin size of 900 $\mu$m was used throughout this chapter, chosen so that the spots could be seen macroscopically, and large amounts of probe could be deposited.

As it had been shown previously in this work that poly-L-lysine slides could be used for immobilisation of antibodies, it was decided to use these initially to determine if whole erythrocytes and erythrocyte membrane (protein) fragments could be deposited and retained. As the poly-L-lysine coating gives the slide surface an overall positive charge, this surface should retain the negatively charged cells. Scott (1991) had previously shown that poly-L-lysine coated microplates could be used to immobilise erythrocytes.

The probes printed for this experiment are described in **Table 6.1.** Whole erythrocytes were washed in PBS, and then re-suspended to represent the haematocrit of whole blood plus doubling dilutions in PBS (starting at 1 in 4). One dilution was used to represent the same number of cells as there was used to prepare the erythrocyte membrane (protein) fragments, which were spotted neat and at one dilution in PBS.

**Table 6.1.** Identity and concentration of probes used in experiment in 6.2.

| Probe identity | Concentration (no. of cells/$\mu l$) |
|---|---|
| Erythrocyte membrane protein fragments (membranes) | $0.14 \times 10^5$ |
| Erythrocyte membrane protein fragments (membranes) | $0.07 \times 10^5$ |
| Whole erythrocytes (Whole RBC) | $0.14 \times 10^5$ |
| Whole erythrocytes (Whole RBC) | $0.28 \times 10^6$ |
| Whole erythrocytes (Whole RBC) | $0.56 \times 10^6$ |
| Whole erythrocytes (Whole RBC) | $1.13 \times 10^6$ |
| Whole erythrocytes (Whole RBC) | $4.5 \times 10^6$ |

Erythrocyte membrane (protein) fragments were extracted from erythrocytes as described in Chapter 2. FITC labelled wheat germ agglutinin (FITC-WGA) (*Triticum vulgaris* lectin) was diluted in PBS-BSA and added as target solution. WGA recognises N-acetyl-D-glucosamine (GlcNAc) and N-acetyl-D-neuraminic acid (NeuAc) residues (Krotkiewska *et al.*, 2002), which are attached to the erythrocyte via glycophorins (reviewed in Schenkel-Brunner, 2000). Values from PBS spots were used to calculate noise.

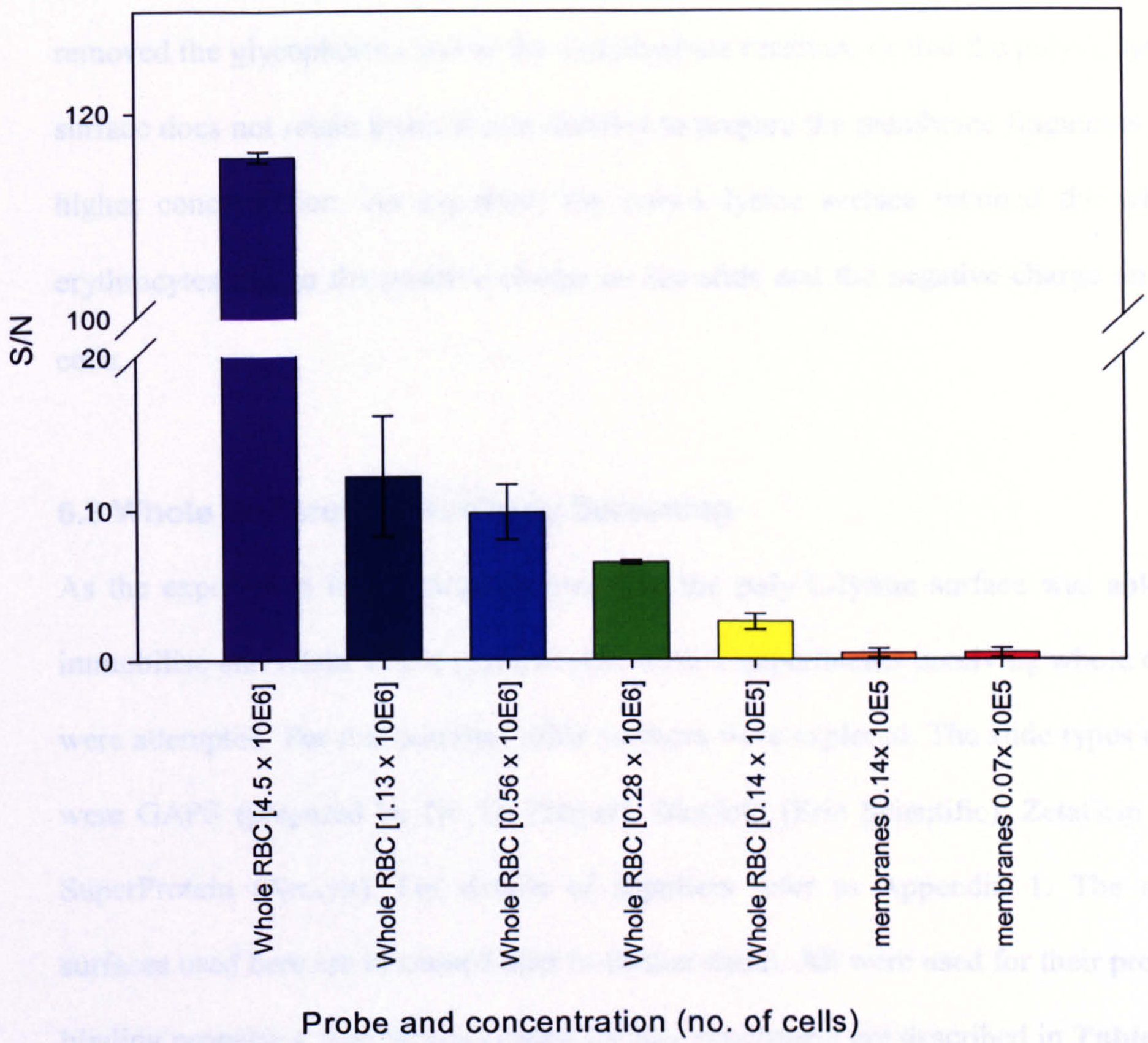



**Figure 6.2**. Results from spotting of whole erythrocytes and membrane protein fragments onto poly-L-lysine slides with FITC WGA 33.3 $\mu$g/ml added as target. Slide type poly-L-lysine, slide reps 1, pins 900 $\mu$m, probes Table 6.1, probe reps 3, SPM E; blocker PBS-BSA, target/volume: FITC WGA/450$\mu$l, incubation time 60min, mixing, scanning method C, data processing method B.

The results (**Figure 6.2.**) show that whole erythrocytes, at the highest concentration, give the strongest S/N value of over 115. The values from the erythrocyte membrane (protein) fragments are very low. Whole erythrocytes spotted at the same concentration used to prepare the membrane (protein) fragments ($0.14 \times 10^5$) gave a low S/N value of 2.49, and the membrane (protein) fragments were lower. This could be due to the concentration of membrane (protein) fragments being too low, or that

FITC WGA does not interact with the membrane proteins and the procedure has removed the glycophorins and/or the carbohydrate residues, or that the poly-L-lysine surface does not retain them. It was decided to prepare the membrane fragments at a higher concentration. As expected, the poly-L-lysine surface retained the whole erythrocytes due to the positive charge on the slide and the negative charge on the cells.

### 6.3 Whole Erythrocyte Antibody Screening

As the experiment in 6.2 demonstrated that the poly-L-lysine surface was able to immobilise and retain whole erythrocytes, further experiments involving whole cells were attempted. For this purpose, other surfaces were explored. The slide types used were GAPS (prepared by Dr. D. Pepper), BioGold (Erie Scientific), ZetaGrip and SuperProtein (Arrayit). For details of suppliers refer to Appendix 1. The slide surfaces used here are discussed later in further detail. All were used for their protein binding properties. The probes printed for this experiment are described in **Table 6.2** and were prepared in PBS.

**Table 6.2.** Identity of probes printed in experiment detailed in 6.3.

| Probe | Probe ID in figures | Concentration |
|---|---|---|
| Group A RhD positive | A RhD pos | $4.5 \times 10^6$/µl |
| Group A RhD negative | A RhD neg | $4.5 \times 10^6$/µl |
| Group B RhD positive | B RhD pos | $4.5 \times 10^6$/µl |
| Group B RhD negative | B RhD neg | $4.5 \times 10^6$/µl |
| Group O RhD positive | O RhD pos | $4.5 \times 10^6$/µl |
| Group O RhD negative | O RhD neg | $4.5 \times 10^6$/µl |
| Synthetic group B antigen | Synthetic B Ag | 1 mg/ml |

Once printed and processed, the target and detection antibodies were used as detailed in **Table 6.3**. Concentration of the monoclonal antibodies prior to dilution is detailed in Chapter 2 (affinity purified antibodies). Prior to the experiment, the ABO group of the human anti-D serum was not known, but it had a potency of 1/256 against group O $R_1r$ cells. The human group O serum contained both anti-A and anti-B at a potency of 1/32 against group $A_1$ and B cells.

**Table 6.3.** Identity of target and detection antibodies used in experiment in 6.3.

| Target Antibody (dilution) | Detection Antibody (concentration) |
|---|---|
| Monoclonal Anti-A LA2 (1/50) | FITC anti-mouse IgM (33.3 $\mu$g/ml) |
| Monoclonal Anti-B LB2 (1/50) | FITC anti-mouse IgM (33.3 $\mu$g/ml) |
| Monoclonal Anti-D LHM169/80 (1/50) | Cy3 anti-human IgG (33.3 $\mu$g/ml) |
| Human anti-D serum (1/10) | Cy3 anti-human IgG (33.3 $\mu$g/ml) |
| Human group O serum (1/10) | Cy3 anti-human IgG (33.3 $\mu$g/ml) |

The results are compiled so that the reactions of all five different target antibodies evaluated on the same slide type are displayed together in **Figures 6.3a-d.** The error bars show the standard error from the replicate spots. **Figure 6.5** shows the results only from monoclonal anti-D (LHM169/80), which gave low but specific results.

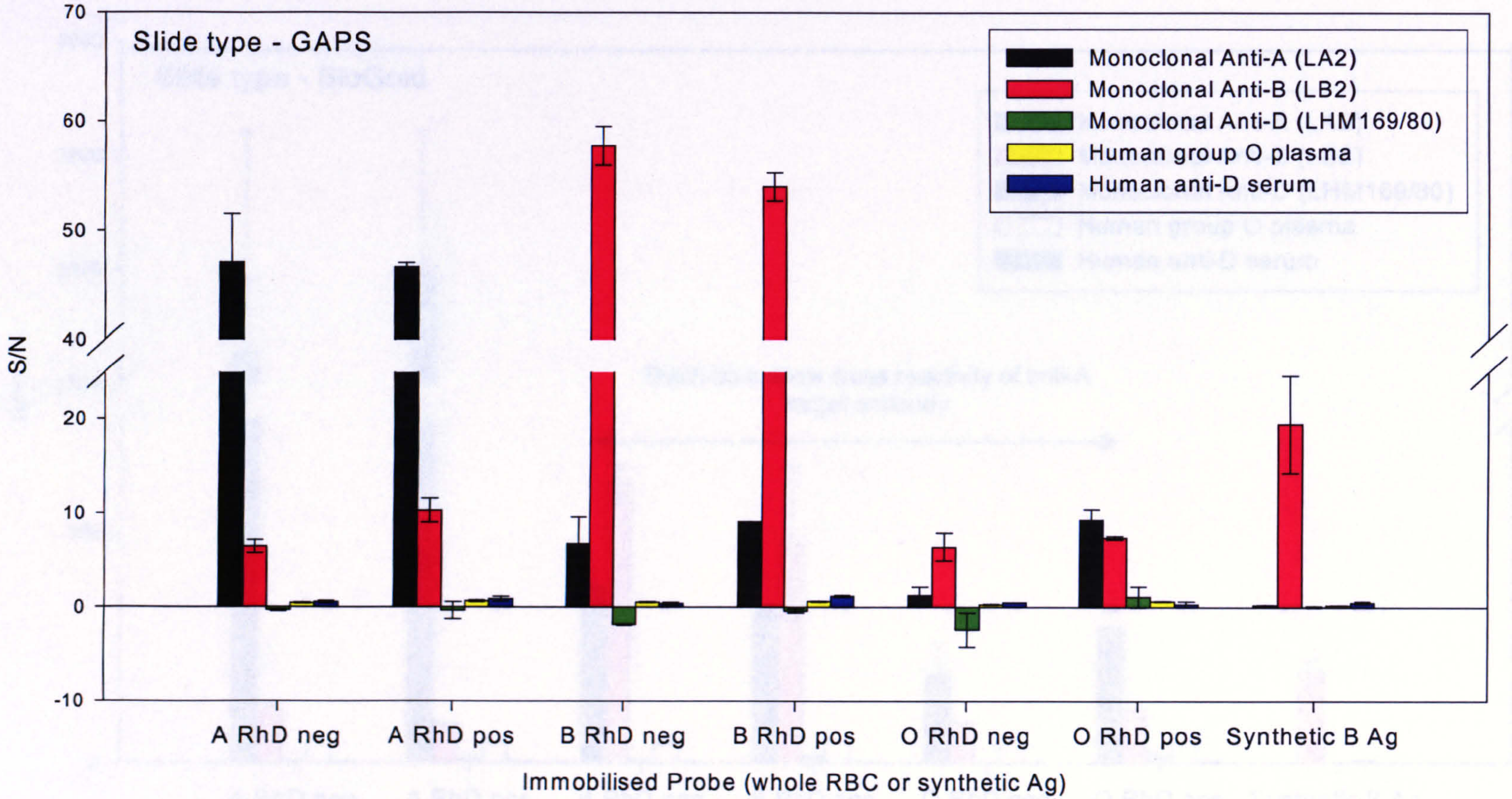



**Figure 6.3a**. Reactions of various target antibodies against immobilised whole erythrocytes or synthetic antigen on GAPS slides. Slide type GAPS, slide reps 1, pins 900 $\mu$m, probes Table 6.2, probe reps 3, SPM E; blocker PBS-BSA, targets detailed in figure, detection detailed in Table 6.3, incubation time 60min, mixing, scanning method C, data processing method B.

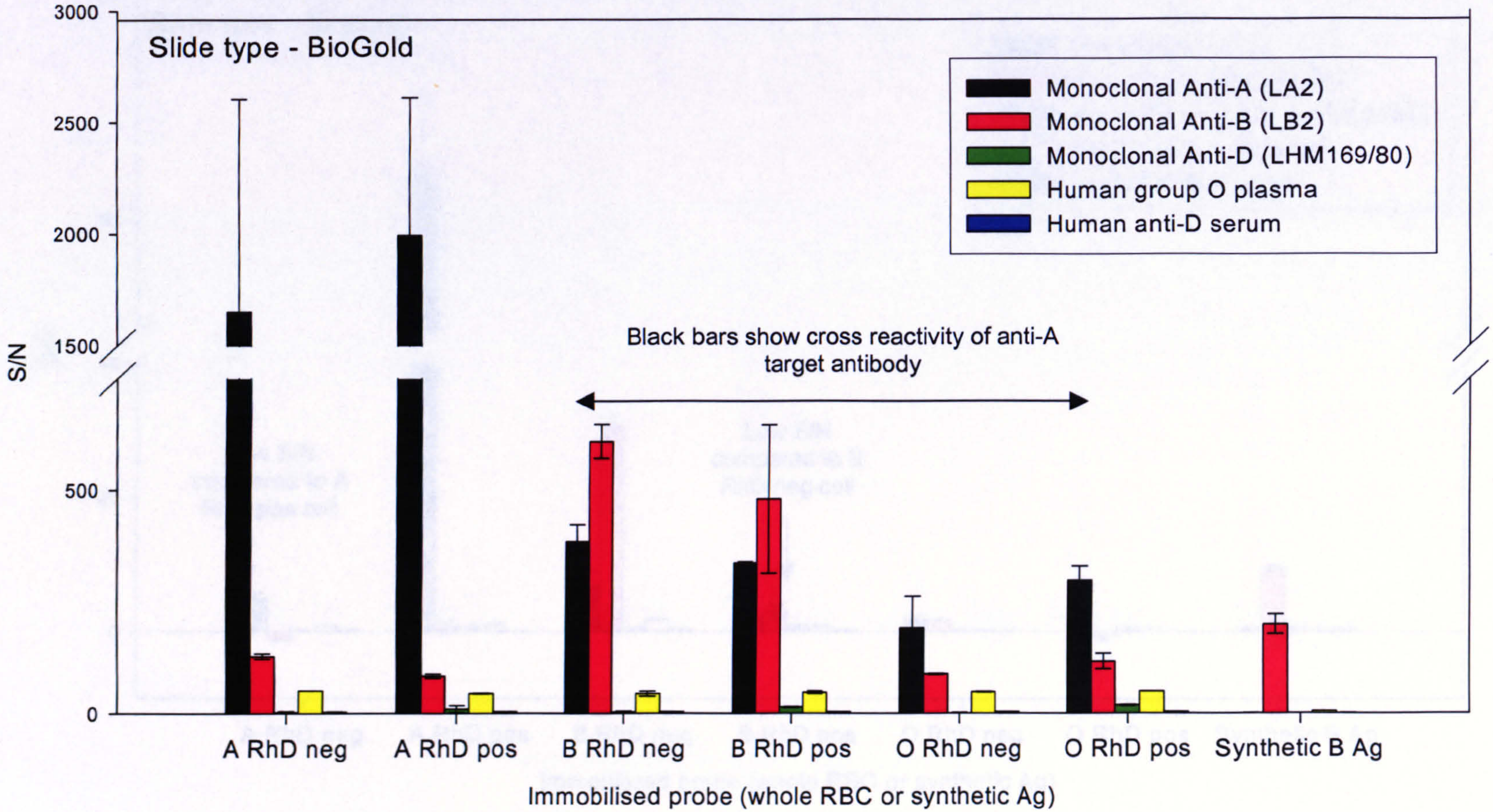



**Figure 6.3b**. Reactions of various target antibodies against immobilised whole erythrocytes or synthetic antigen on BioGold slides. Slide type BioGold, slide reps 1, pins 900 $\mu$m, probes Table 6.2, probe reps 3, SPM E; blocker PBS-BSA, targets detailed in figure, detection detailed in Table 6.3, incubation time 60min, mixing, scanning method C, data processing method B.

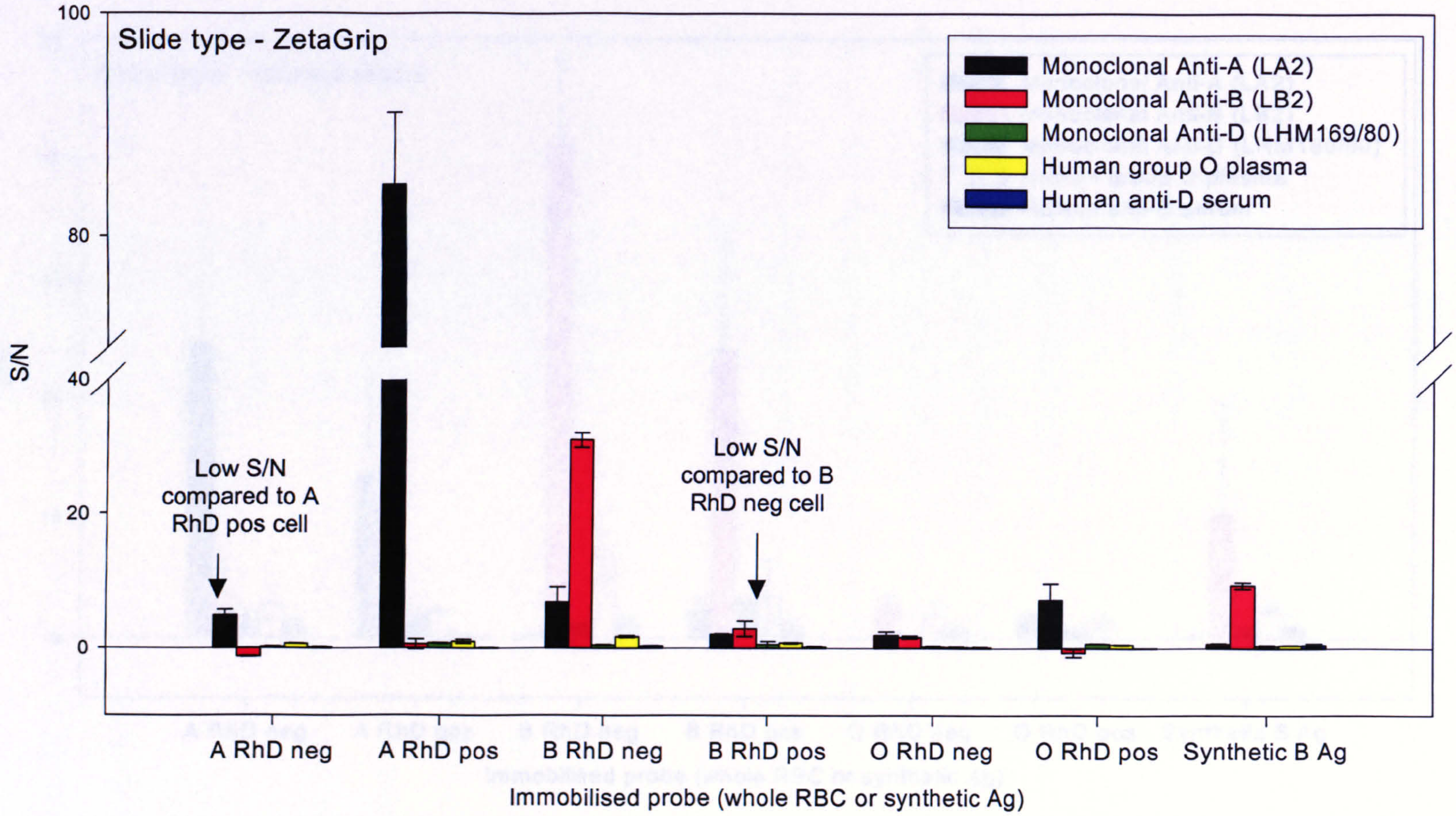



**Figure 6.3c**. Reactions of various target antibodies against immobilised whole erythrocytes or synthetic antigen on ZetaGrip slides. Slide type ZetaGrip, slide reps 1, pins 900 $\mu$m, probes Table 6.2, probe reps 3, SPM E; blocker PBS-BSA, targets detailed in figure, detection detailed in Table 6.3, incubation time 60min, mixing, scanning method C, data processing method B.

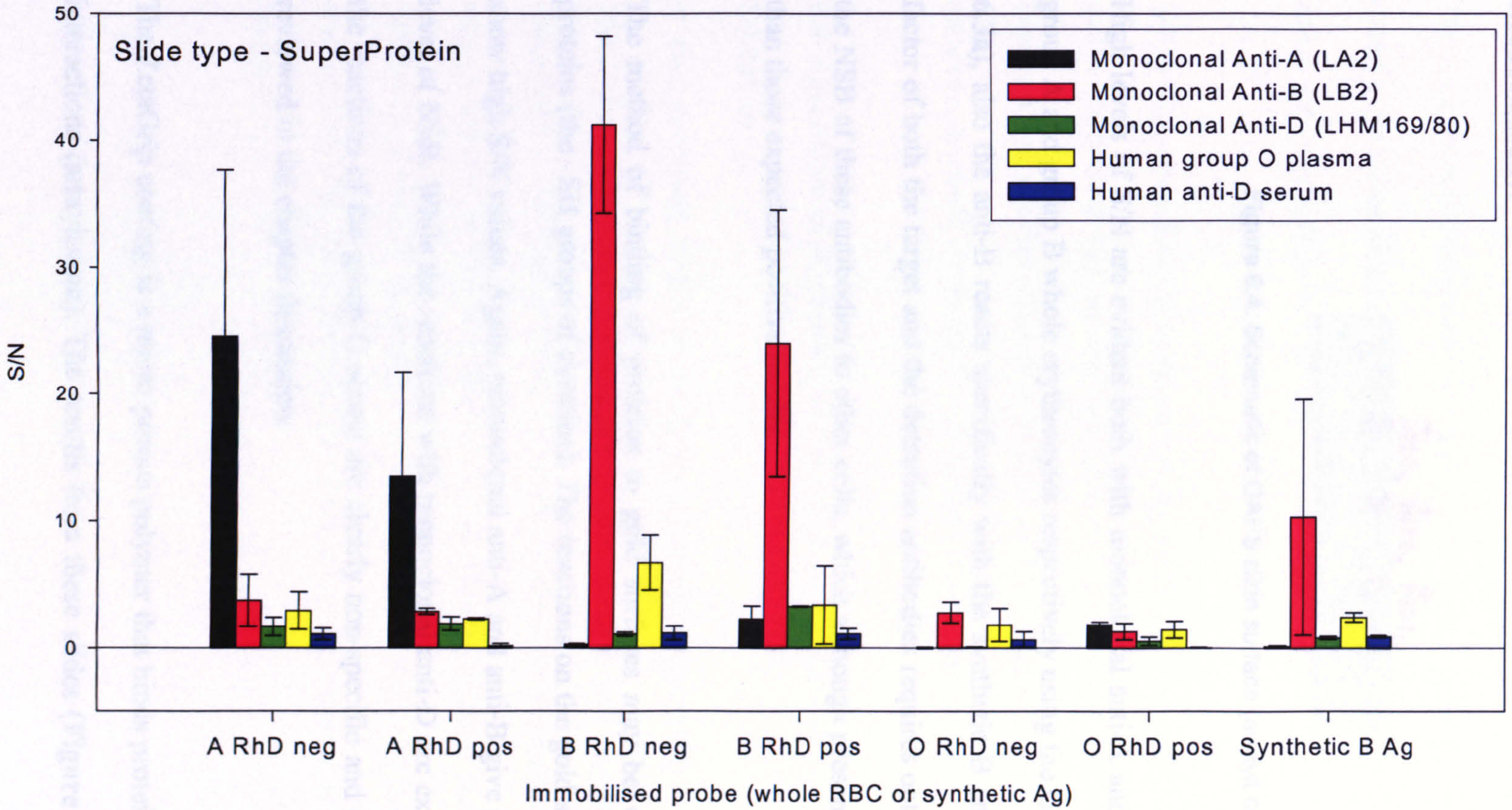



**Figure 6.3d**. Reactions of various target antibodies against immobilised whole erythrocytes or synthetic antigen on SuperProtein slides. Slide type SuperProtein, slide reps 1, pins 900 $\mu$m, probes Table 6.2, probe reps 3, SPM E; blocker PBS-BSA, targets detailed in figure, detection detailed in Table 6.3, incubation time 60min, mixing, scanning method C, data processing method B.

The GAPS (γ-aminopropylsilane) surface has positively charged amino groups ($NH_3^+$) as demonstrated in **Figure 6.4**, which will bind negatively charged molecules electrostatically.


**Figure 6.4**. Schematic of GAPS slide surface (arrayit.com).

High levels of S/N are evident both with monoclonal anti-A and anti-B against the group A and group B whole erythrocytes respectively using the GAPS slides (**Figure 6.3a**), also the anti-B reacts specifically with the synthetic B antigen. The dilution factor of both the target and the detection antibodies requires optimisation to reduce the NSB of these antibodies to other cells, which although present are much reduced than those expected positive results.

The method of binding of proteins to gold surfaces may be via thiol groups of proteins (the –SH groups of cysteine). The reactions on the gold slides (**Figure 6.3b**) show high S/N values. Again, monoclonal anti-A and anti-B give good results, with a level of NSB. While the reactions with monoclonal anti-D are examined again later, the reactions of the group O serum are clearly non-specific and reasons for this are reviewed in the chapter discussion.

The ZetaGrip coating is a micro porous polymer that binds proteins via hydrophobic interactions (arrayit.com). The results from these slides (**Figure 6.3c**) do not show

the expected pattern. Both monoclonal anti-A and anti-B react with only one of two cells, which should be positive.

The SuperProtein surface also binds proteins via hydrophobic interactions. There are specific reactions **(Figure 6.3d)** with the monoclonal anti-A and anti-B, with the anti-B also interacting with the synthetic B antigen.

When each specificity was examined separately, it was noted that the monoclonal anti-D gave weak but specific reactions on the BioGold surface. The bar chart in **Figure 6.5** shows that although giving a low S/N value, it is clear the reactions of the monoclonal anti-D are specific on the gold surface, but not on any other surface.

## 6.4 Retention of Erythrocyte Membrane Protein Fragments on Various Surfaces

In the experiment in 6.2, an erythrocyte membrane (protein) fragment preparation was spotted, but possibly at too low a concentration for detection. It was also not determined if the fragments were actually retained on the surface. This experiment was performed to examine the extent to which different surfaces could retain membrane (protein) fragments, using fragments prepared from FITC labelled erythrocytes. The probe erythrocyte fragments were prepared at two concentrations **(Table 6.4)**.

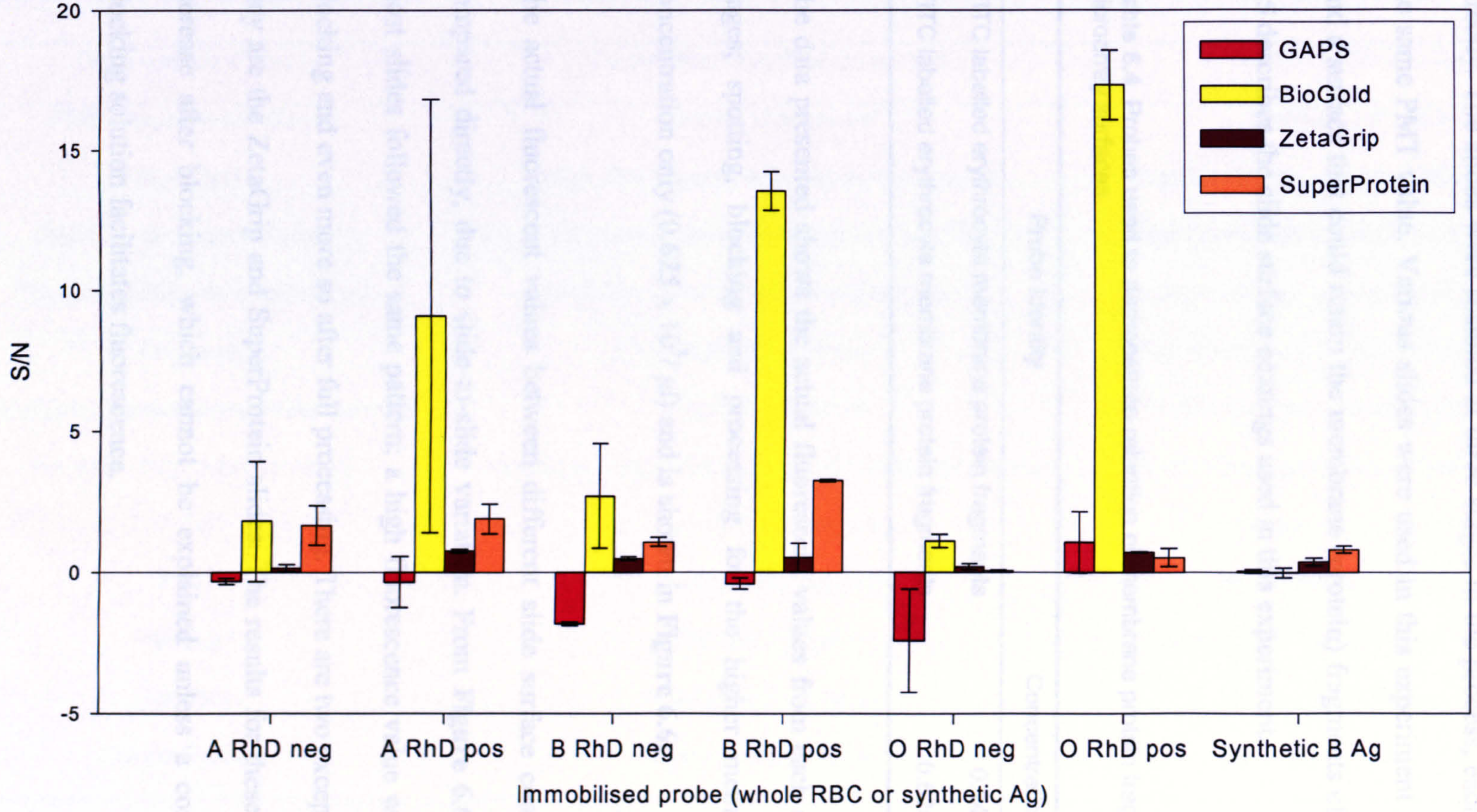



**Figure 6.5**. Reactions of monoclonal anti-D LHM169/80 only, against immobilised whole erythrocytes or synthetic antigen, on all slide types. Slide type various, slide reps 1, pins 900 $\mu$m, probes Table 6.2, probe reps 3, SPM E; blocker PBS-BSA, target monoclonal anti-D LHM169/80, detection detailed in Table 6.3, incubation time 60min, mixing, scanning method C, data processing method B.

Using these preparations, the slides were processed routinely but with no detection solution added, as the fragments were FITC labelled and would be detectable directly. The slides were scanned at three stages in the process, each slide always at the same PMT value. Various slides were used in this experiment in an attempt to find a surface that could retain the membrane (protein) fragments effectively. **Table 6.5** describes the slide surface coatings used in this experiment.

**Table 6.4**. Probes used to demonstrate retention of membrane protein fragments on various microarray surfaces.

| Probe Identity | Concentration (cells per $\mu$l) |
|---|---|
| FITC labelled erythrocyte membrane protein fragments | $0.14 \times 10^5$ |
| FITC labelled erythrocyte membrane protein fragments | $0.625 \times 10^5$ |

The data presented shows the actual fluorescent values from each slide type at the stages; spotting, blocking and processing for the higher membrane fragment concentration only ($0.625 \times 10^5$/ $\mu$l) and is shown in **Figure 6.6**.

The actual fluorescent values between different slide surface coatings cannot be compared directly, due to slide-to-slide variation. From **Figure 6.6** it is clear that most slides followed the same pattern; a high fluorescence value which drops after blocking and even more so after full processing. There are two exceptions to this and they are the ZetaGrip and SuperProtein slides. The results for these two slide types increase after blocking, which cannot be explained unless a component of the blocking solution facilitates fluorescence.

**Table 6.5.** Slides used in experiment to compare retention of erythrocyte membrane proteins fragments on different microarray surfaces.

| Slide surface coating | Description of surface properties | Action of attachment |
|---|---|---|
| PEG (polyethylene glycol) | Blocking compound | Blocking compound |
| Poly-L-lysine | High MW polymer with free amino groups. | Positive charge – electrostatic adsorption |
| GAPS (*γ*-aminopropylsilane) | Low MW silane, converts silanols into amino groups. | Positive charge – electrostatic adsorption |
| PEI (poly (ethylene isopthalate)) | High MW polymer with free amino groups | Positive charge – electrostatic adsorption |
| Polybrene | High MW polymer | Positive charge – electrostatic adsorption |
| pDADMAC (polydiallyldimethylammonium chloride) | High MW polymer | Positive charge – electrostatic adsorption |
| PAMAM (poly (amidoamine)) | Dendrimer - High MW polymer with free amino groups | Positive charge – electrostatic adsorption |
| P4VP | Hydrophobic polymer | Hydrophobic interactions |
| P2VP | Hydrophobic polymer | Hydrophobic interactions |
| BioGold | Thin metal coating | Reacts with free thiols (on cysteine) |
| ZetaGrip | Hydrophobic polymer | Hydrophobic interactions |
| SuperProtein | Hydrophobic polymer | Hydrophobic interactions |

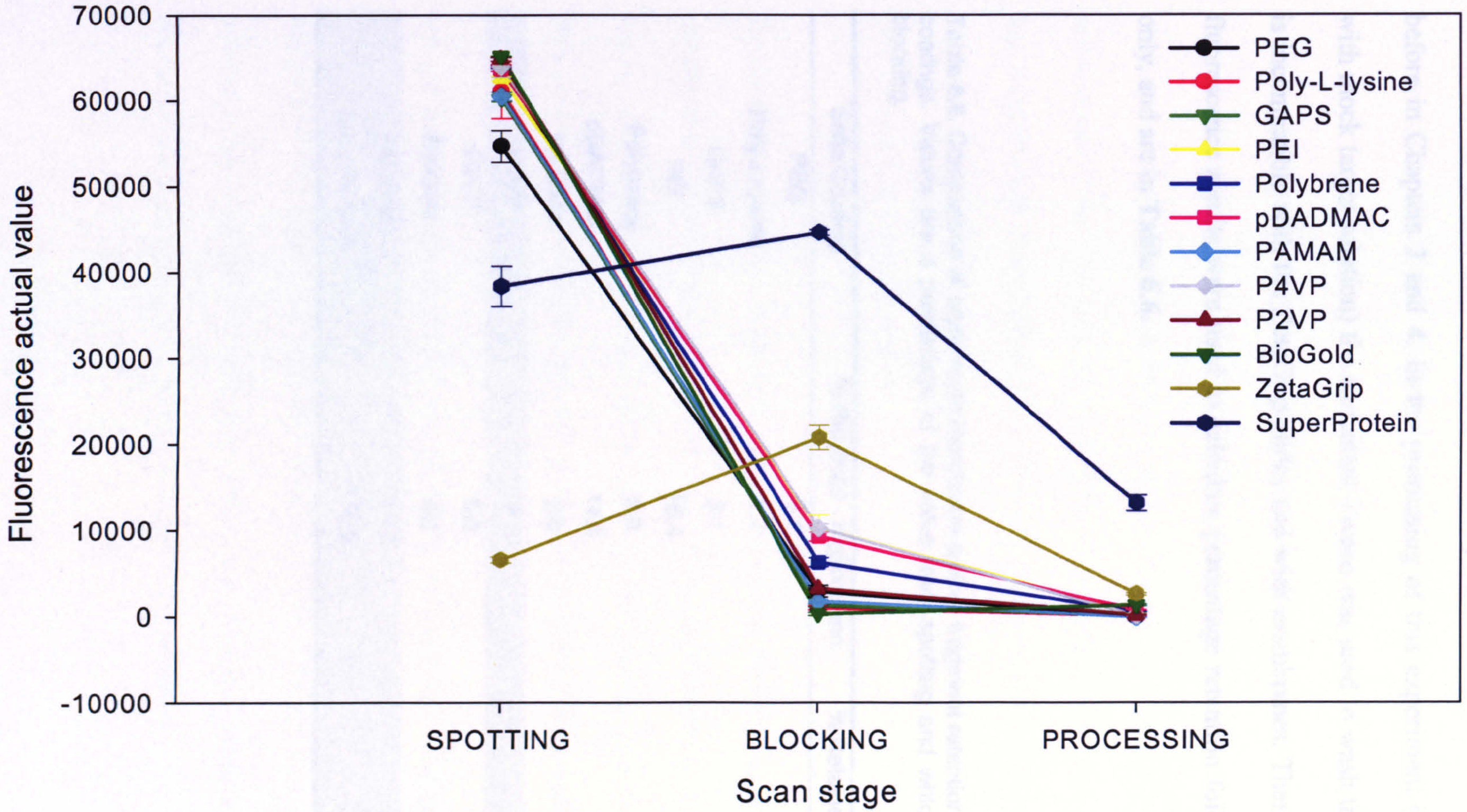



**Figure 6.6**. Line plot, showing results of experiment examining retention of erythrocyte membrane protein fragments on various slide coatings. Slide type various, slide reps 1, pins 900 $\mu$m, probes Table 6.4, probe reps 3, SPM E; blocker PBS-BSA, target not applicable, detection FITC labelled erythrocyte fragments, incubation time 60min, mixing, scanning method C, data processing method B.

The BioGold slides showed good results when using whole erythrocytes, but when using erythrocyte membrane (protein) fragments the effect is opposite. It is clear that different surfaces are more suited to certain purposes. This has been demonstrated before in Chapters 3 and 4. In the processing of this experiment (after incubation with mock target solution) the surfactant Tween was used to wash the slides. Tween is incompatible with the ZetaGrip slides and with membranes. Therefore, the actual fluorescence results were used to calculate percentage retention following blocking only, and are in **Table 6.6**.

**Table 6.6**. Comparison of erythrocyte membrane protein fragment retention on different slide coatings. Values are a percentage of the value from spotting and which remained after blocking.

| Slide Coating | % Retained - higher conc. | % Retained - lower conc. |
|---|---|---|
| PEG | 5.4 | 13.1 |
| Poly-L-lysine | 1.7 | 2.7 |
| GAPS | 2.1 | 3.3 |
| PEI | 16.4 | 17.7 |
| Polybrene | 9.8 | 10.1 |
| pDADMAC | 14.6 | 9.8 |
| PAMAM | 2.9 | 4.9 |
| P4VP | 16.0 | 19.5 |
| P2VP | 5.0 | 8.6 |
| BioGold | 0.0 | 0.3 |
| ZetaGrip | 315.6 | 252.1 |
| SuperProtein | 116.3 | 154.2 |

The results highlighted in grey (**Table 6.6**) show that FITC labelled erythrocyte membrane (protein) fragments were bound more to those slide coatings. These slides were selected for further experiments. The BioGold slides were also selected as they had previously shown to retain whole erythrocytes on their surface.

## 6.5 Antibody Screening Using Whole Erythrocytes and Membrane Protein Fragments on Selected Slide Surface Coatings

Experiments performed thus far have shown that gold coated slides can bind whole erythrocytes to successfully perform antibody screening, and that P4VP, ZetaGrip and SuperProtein coated slides can retain erythrocyte membrane (protein) fragments. The following experiment was performed to determine if antibody screening could be performed on these slide types.

Erythrocyte membrane fragments were prepared at the highest concentration (as described in Chapter 2 and in previous experiment) and spotted onto the various slide types. Also spotted were the whole erythrocytes used to prepare the membrane fragments, where available, and the antibodies that were later used as targets. The target antibodies were printed to give a positive control for the detection antibody. Target and detection antibodies are described in **Table 6.7.** The anti-A and anti-B had previously been shown to detect whole erythrocytes in a microarray format. The antigens to which they are directed are carbohydrates. The anti-K is directed to the K antigen, a glycoprotein molecule (see **Table 1.4**), which is close to the erythrocyte membrane and, therefore, may be less accessible for detection.

**Table 6.7.** Identity of target and detection antibodies used in experiment in 6.5.

| Target antibody (dilution) | Detection antibody (concentration) |
|---|---|
| Monoclonal anti-A LA2 (1/50) | FITC anti-mouse IgM (33.3 $\mu$g/ml) |
| Monoclonal anti-D LHM169/80 (1/50) | Cy3 anti-human IgG (33.3 $\mu$g/ml) |
| Monoclonal anti-K (MS56)(1/50) | Cy3 anti-human IgM (33.3 $\mu$g/ml) |

The results from this experiment are presented in **Figures 6.7a-d.** The fluorescence values are used as data points in these figures, and are arranged in a descending numerical order to aid the interpretation of the results. The values given are the average of the duplicate spots for each sample (no error bars).

When scanning the slides in this experiment it was noticed that there were many black holes on the scan images. These can arise where blocking is inadequate and leads to NSB around the spot, or perhaps where there is free fluorescent label in the detection solution (i.e. the post-labelling washing was not sufficient). As some of these were present, the actual fluorescence value is used rather than a S/N ratio. In the charts, MP is the erythrocyte membrane (protein) fragments and WC refers to the whole cells.

**Figure 6.7a** shows the results from the P4VP slides. Where anti-A was used, the reactions are greater with the control anti-A, the $A_1$ and $A_2B$ membrane protein fragments, but not largely greater than the other fragments present. The anti-D results are not as expected as reactions with RhD positive probes are low.

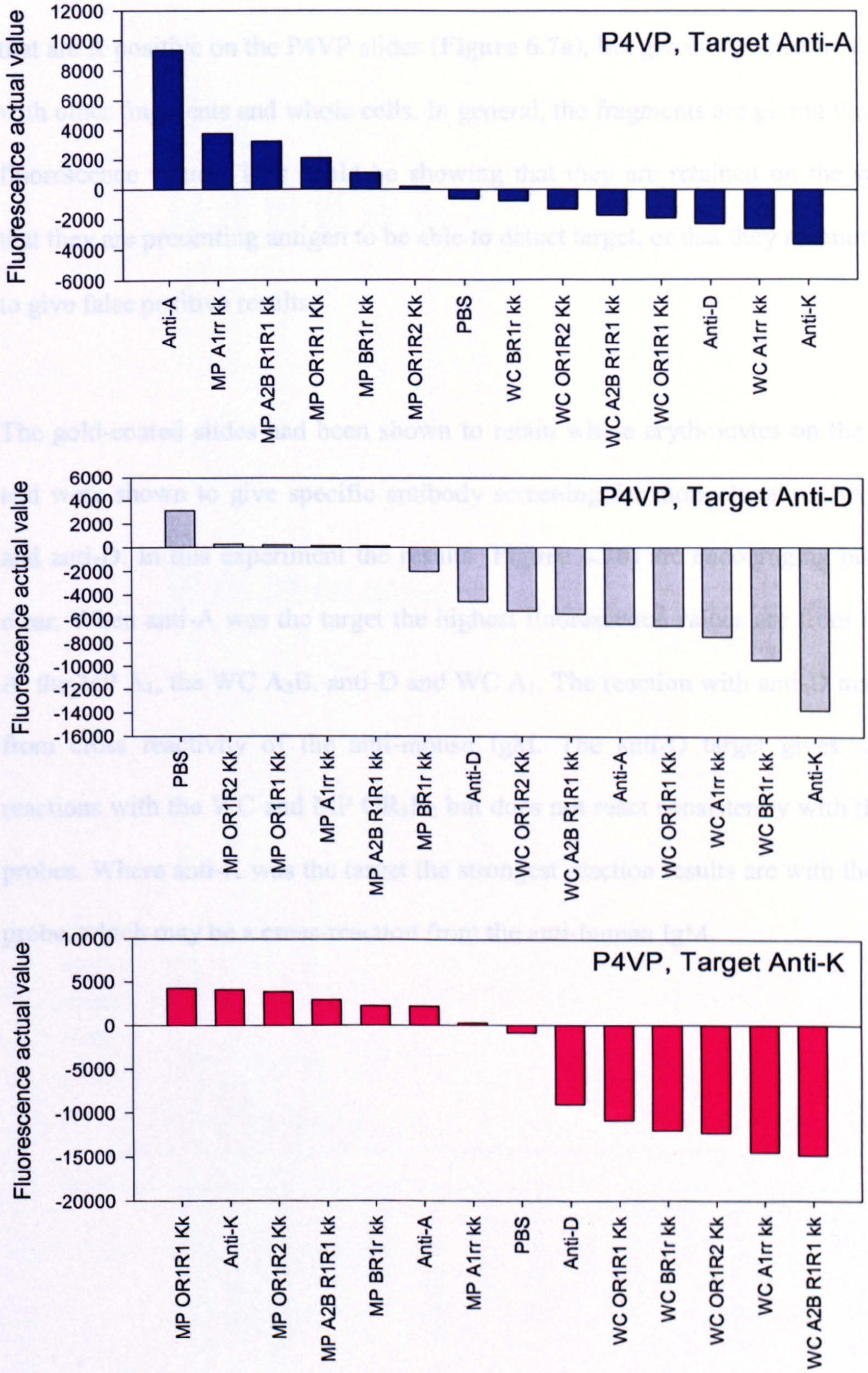



**Figure 6.7a**. Results from P4VP slides with target antibodies anti-A, anti-D and anti-K. Slide type P4VP, slide reps 1, pins 900 $\mu$m, probes detailed on x-axis, probe reps 2, SPM E; blocker PBS-BSA, target/detection Table 6.7, incubation time 60min, mixing, scanning method C, data processing method B.

The anti-K gives stronger reactions with some of the membrane (protein) fragments that are K positive on the P4VP slides (**Figure 6.7a**), but gives non-specific reactions with other fragments and whole cells. In general, the fragments are giving the greater fluorescence values. This could be showing that they are retained on the surfaces, that they are presenting antigen to be able to detect target, or that they are more likely to give false positive results.

The gold-coated slides had been shown to retain whole erythrocytes on the surface and were shown to give specific antibody screening for monoclonal anti-A, anti-B and anti-D. In this experiment the results (**Figure 6.7b**) are encouraging but not as clear. When anti-A was the target the highest fluorescence values are from the anti-A, the MP $A_1$, the WC $A_2B$, anti-D and WC $A_1$. The reaction with anti-D may result from cross reactivity of the anti-mouse IgM. The anti-D target gives strongest reactions with the WC and MP $OR_1R_2$ but does not react consistently with the other probes. Where anti-K was the target the strongest reaction results are with the anti-A probe, which may be a cross-reaction from the anti-human IgM.

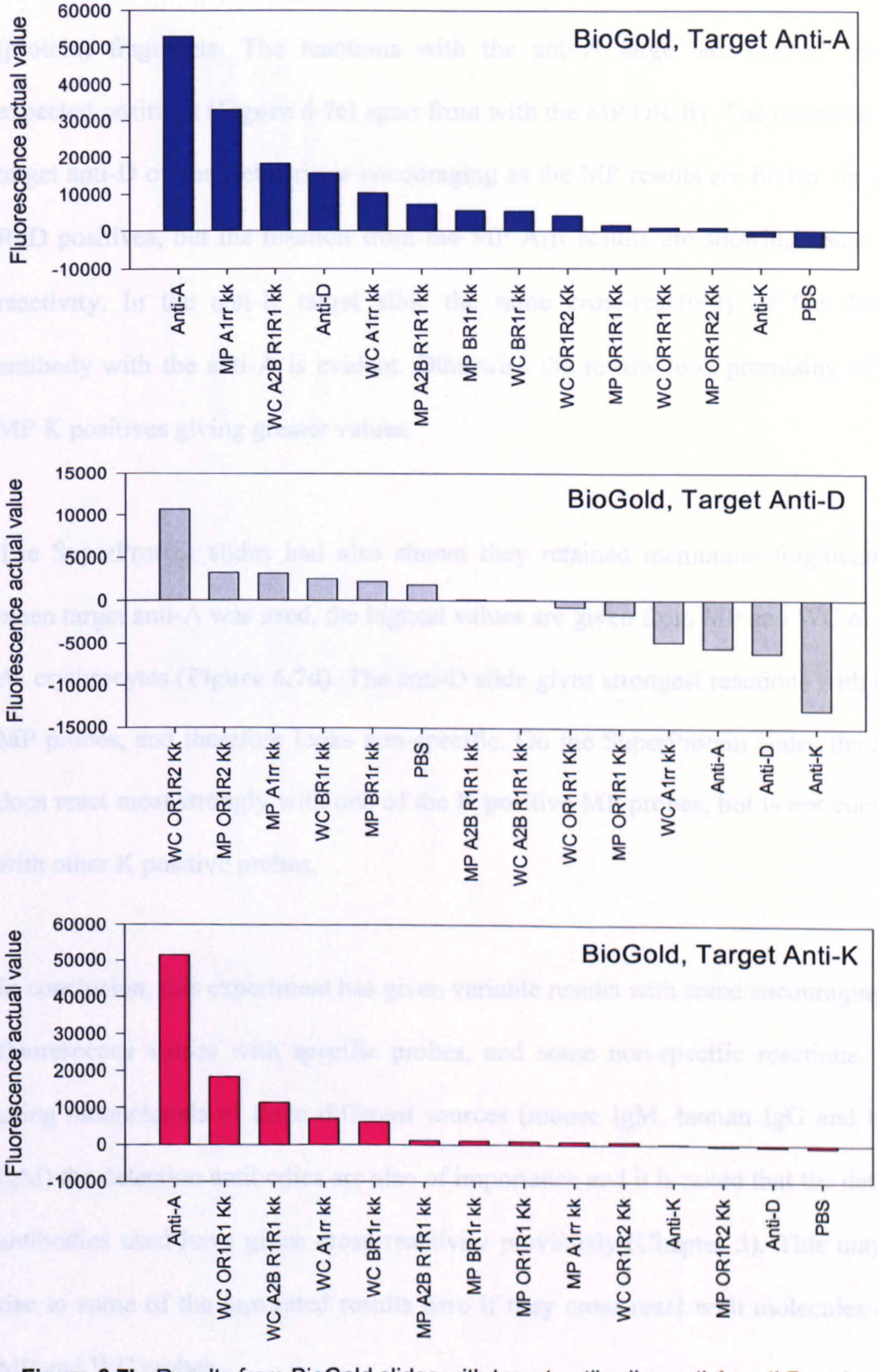



**Figure 6.7b**. Results from BioGold slides with target antibodies anti-A, anti-D and anti-K. Slide type BioGold, slide reps 1, pins 900 $\mu$m, probes detailed on x-axis, probe reps 2, SPM E; blocker PBS-BSA, target/detection Table 6.7, incubation time 60min, mixing, scanning method C, data processing method B.

Results from the ZetaGrip slides had shown that the surface retained membrane (protein) fragments. The reactions with the anti-A target are highest with the expected positives (**Figure 6.7c**) apart from with the MP $OR_1R_1$. The reactions of the target anti-D on the ZetaGrip is encouraging as the MP results are higher for all the RhD positives, but the reaction from the MP $A_1rr$ results are showing some cross-reactivity. In the anti-K target slide the same cross-reactivity of the detection antibody with the anti-A is evident. Otherwise the results look promising with the MP K positives giving greater values.

The SuperProtein slides had also shown they retained membrane fragments, and when target anti-A was used, the highest values are given from MP and WC of group $A_1$ erythrocytes (**Figure 6.7d**). The anti-D slide gives strongest reactions with all the MP probes, and therefore looks non-specific. On the SuperProtein slides the anti-K does react most strongly with one of the K positive MP probes, but is not consistent with other K positive probes.

In conclusion, this experiment has given variable results with some encouraging high fluorescence values with specific probes, and some non-specific reactions. When using monoclonals of three different sources (mouse IgM, human IgG and human IgM) the detection antibodies are also of importance and it is noted that the detection antibodies used have given cross reactivity previously (Chapter 3). This may give rise to some of the unwanted results here if they cross-react with molecules of the MP and WC probes.

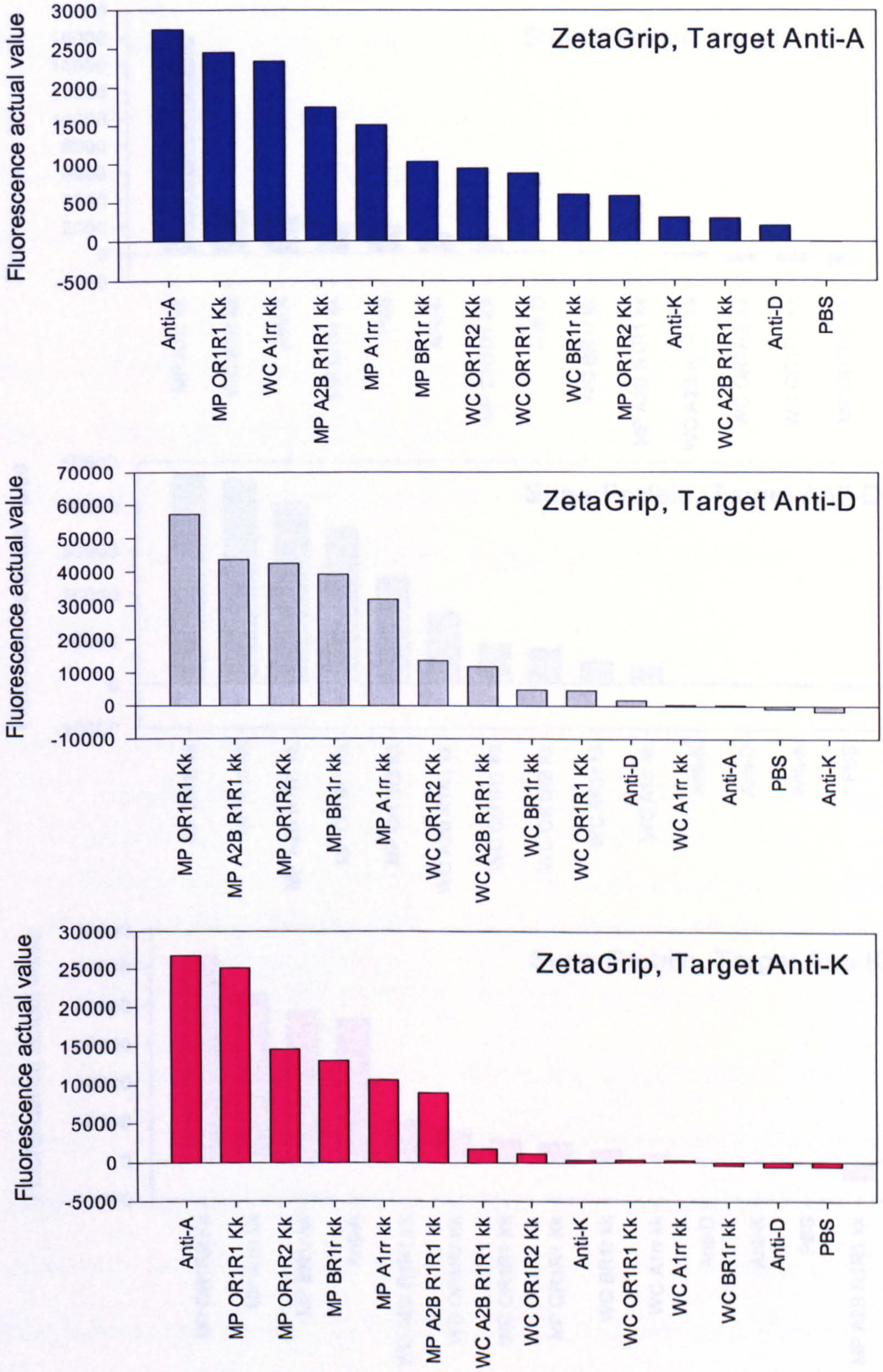



**Figure 6.7c**. Results from ZetaGrip slides with target antibodies anti-A, anti-D and anti-K. Slide type ZetaGrip, slide reps 1, pins 900 $\mu$m, probes detailed on x-axis, probe reps 2, SPM E; blocker PBS-BSA, target/detection Table 6.7, incubation time 60min, mixing, scanning method C, data processing method B.

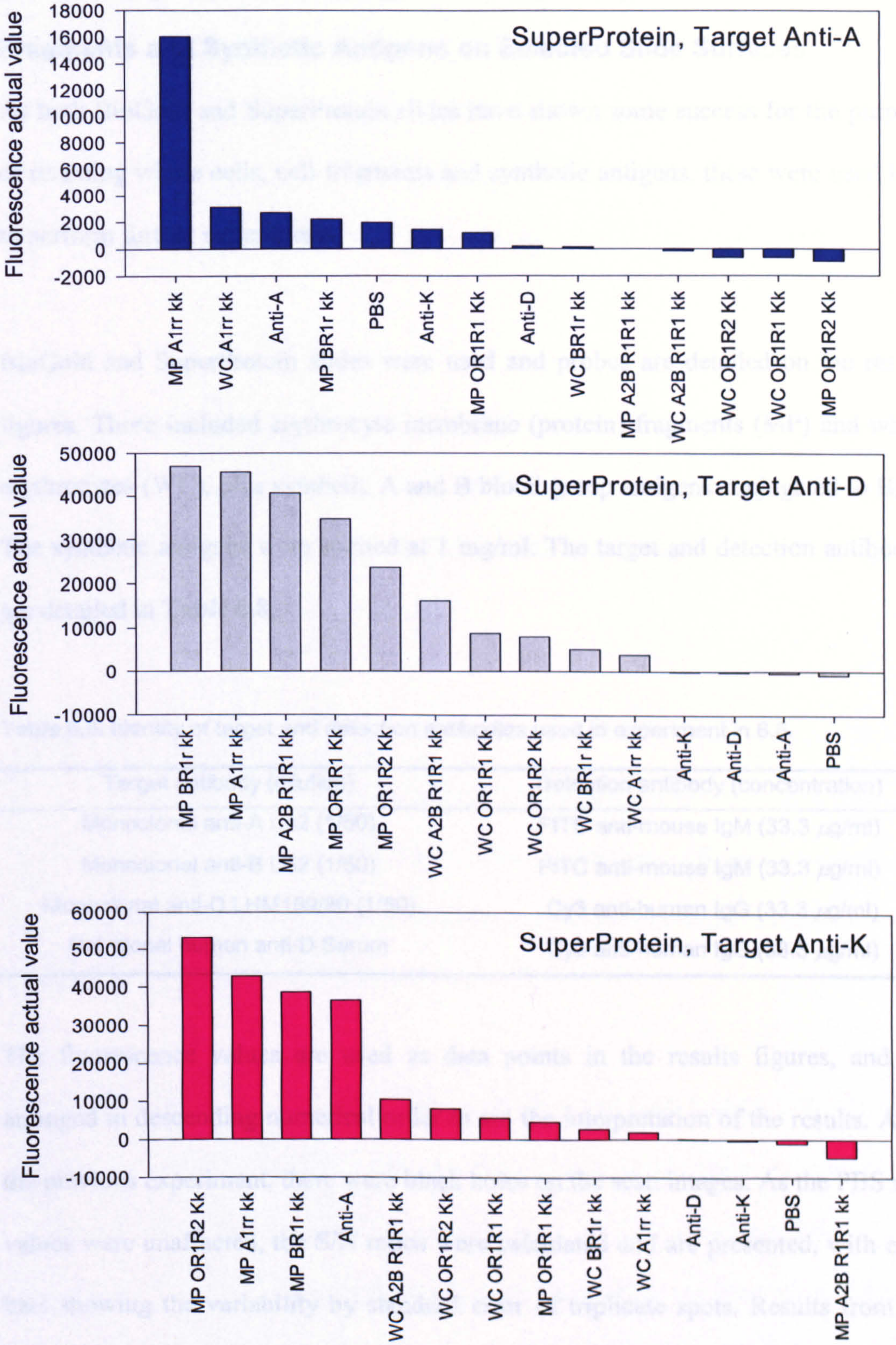



**Figure 6.7d**. Results of SuperProtein slides with target antibodies anti-A, anti-D and anti-K. Slide type SuperProtein, slide reps 1, pins 900 $\mu$m, probes detailed on x-axis, probe reps 2, SPM E; blocker PBS-BSA, target/detection Table 6.7, incubation time 60min, mixing, scanning method C, data processing method B.

## 6.6 Antibody Screening Using Whole Erythrocytes, Membrane Protein Fragments and Synthetic Antigens on Selected Slide Surfaces

As both BioGold and SuperProtein slides have shown some success for the purpose of retaining whole cells, cell fragments and synthetic antigens, these were used here to perform further experiments.

BioGold and SuperProtein slides were used and probes are detailed on the results figures. These included erythrocyte membrane (protein) fragments (MP) and whole erythrocytes (WC), plus synthetic A and B blood group antigens conjugated to BSA. The synthetic antigens were spotted at 1 mg/ml. The target and detection antibodies are detailed in **Table 6.8.**

**Table 6.8.** Identity of target and detection antibodies used in experiment in 6.6.

| Target antibody (dilution) | Detection antibody (concentration) |
|---|---|
| Monoclonal anti-A LA2 (1/50) | FITC anti-mouse IgM (33.3 $\mu$g/ml) |
| Monoclonal anti-B LB2 (1/50) | FITC anti-mouse IgM (33.3 $\mu$g/ml) |
| Monoclonal anti-D LHM169/80 (1/50) | Cy3 anti-human IgG (33.3 $\mu$g/ml) |
| Polyclonal human anti-D Serum | Cy3 anti-human IgG (33.3 $\mu$g/ml) |

The fluorescence values are used as data points in the results figures, and are arranged in descending numerical order to aid the interpretation of the results. As in the previous experiment, there were black holes on the scan images. As the PBS spot values were unaffected, the S/N ratios were calculated and are presented, with error bars showing the variability by standard error of triplicate spots. Results from the BioGold and SuperProtein slides against the same target antibody are presented in the same figure.

The results from the anti-A target when evaluated on both BioGold and SuperProtein slides are shown in **Figure 6.8a.** The BioGold slide gives the highest S/N ratio against both the WC and MP group $A_1$, but the ratio is less than that demonstrated in **Figure 6.3b.** In **Figure 6.3b** there also was some cross-reactivity. The SuperProtein slide shows the highest S/N with MP $A_1$ but the next in descending order is an MP group O.

The results from the anti-B target when evaluated on both BioGold and SuperProtein slides are shown in **Figure 6.8b.** The gold surface shows again that it retains whole erythrocytes, as there are specific reactions with both group B WC probes and the anti-B reacts with the synthetic B antigen.

The monoclonal anti-D (LHM169/80) reacts specifically with the RhD positive whole cells on the gold surface, although S/N levels are low (3-6) **(Figure 6.8c).** The monoclonal anti-D on the SuperProtein surface shows quite different results **(Figure 6.8c)** where all membrane (protein) fragments give similar results and reactions are, therefore non-specific.

Again on the gold slides, the polyclonal anti-D reacts with highest S/N with RhD positive whole cells **(Figure 6.8d),** however values from group $A_1$ and B RhD negative whole cells are giving a notable level of S/N (positives 16-22, while negatives 7-10). In the same figure the SuperProtein slides are showing highest S/N values with RhD positive membrane (protein) fragments, although group $A_1$ MP are showing S/N of 3.

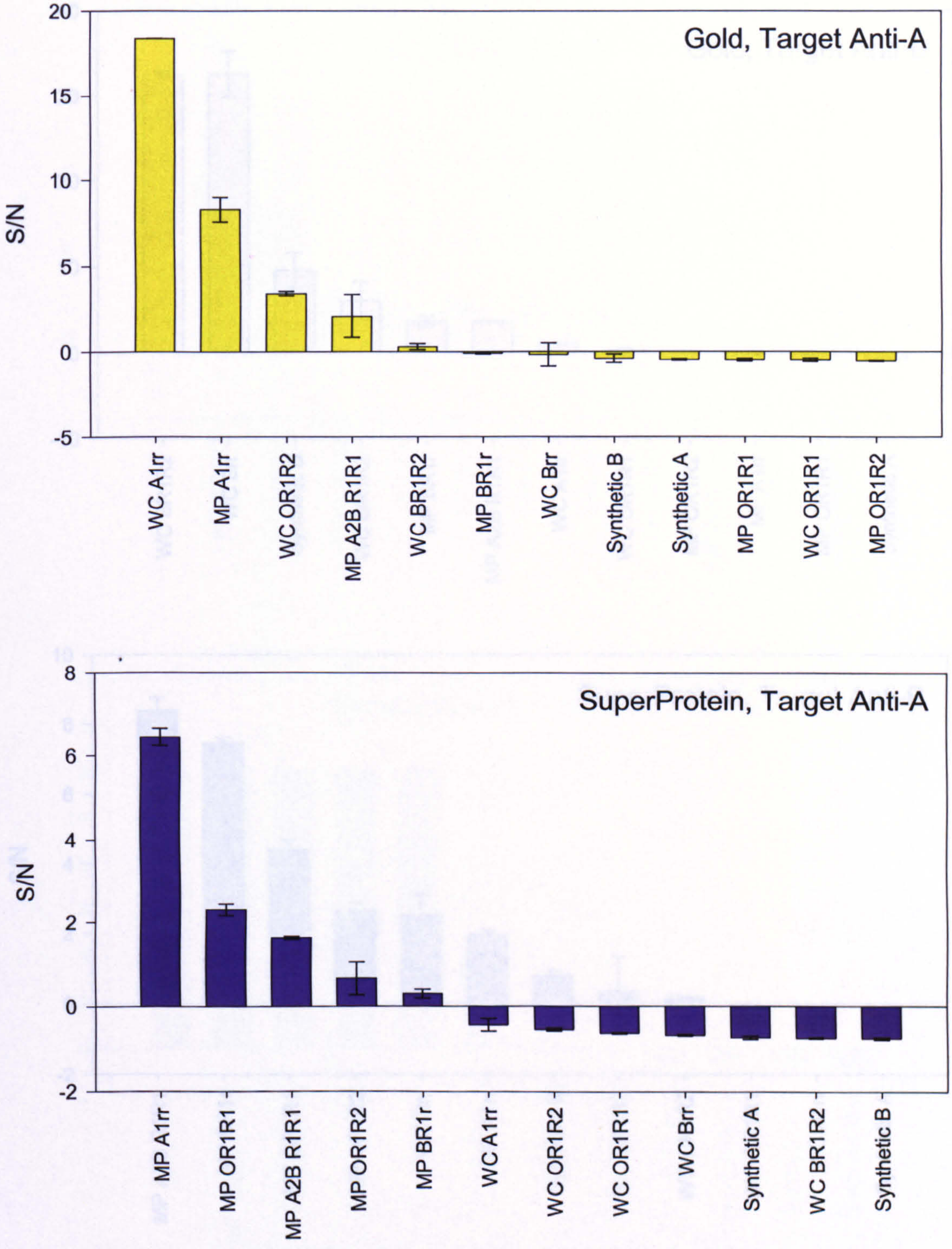



**Figure 6.8a**. Results of BioGold and SuperProtein slides against target anti-A. Slide type detailed in figure, slide reps 1, pins 900 $\mu$m, probes detailed on x-axis, probe reps 3, SPM E; blocker PBS-BSA, target/detection Table 6.8, incubation time 60min, mixing, scanning method C, data processing method B.

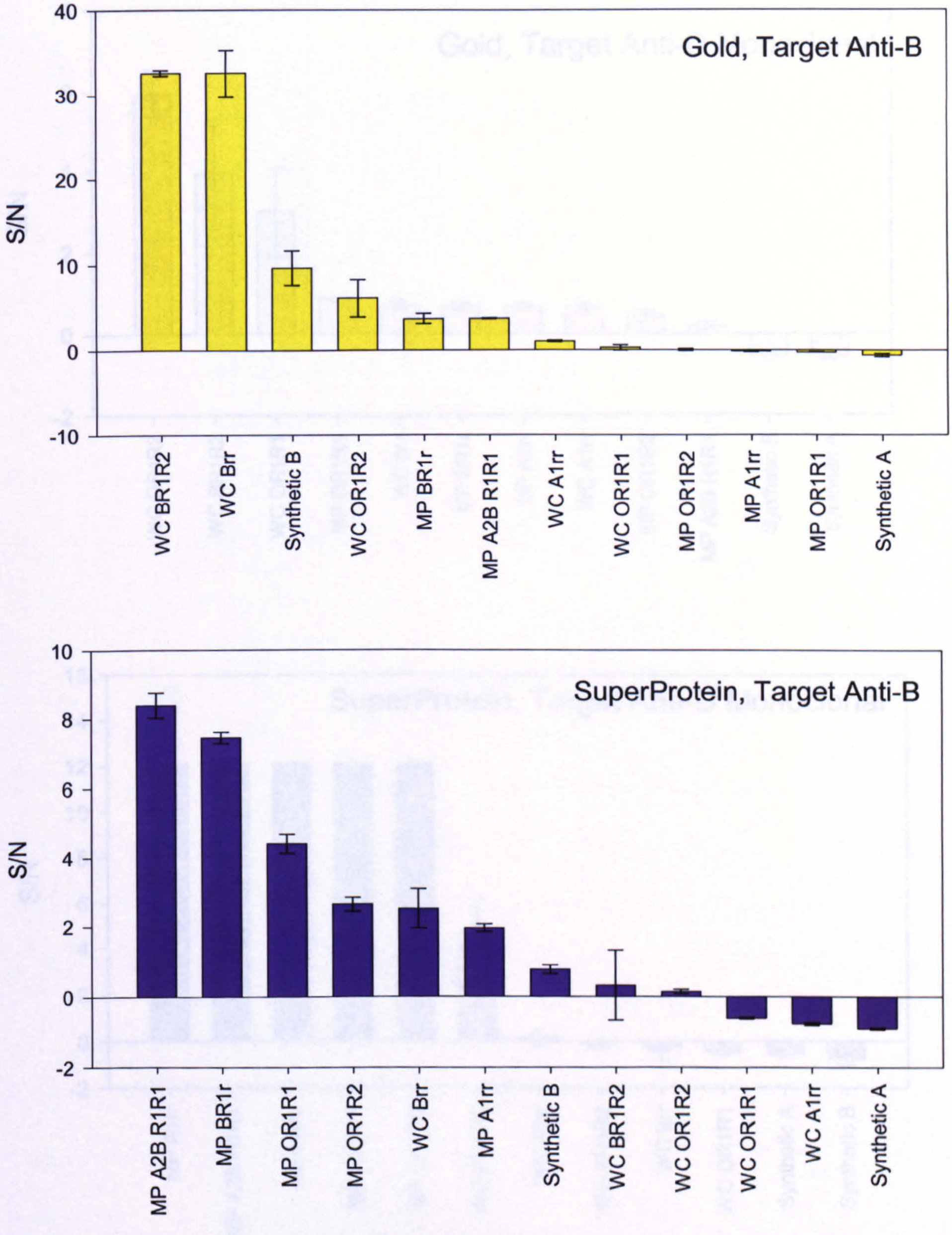
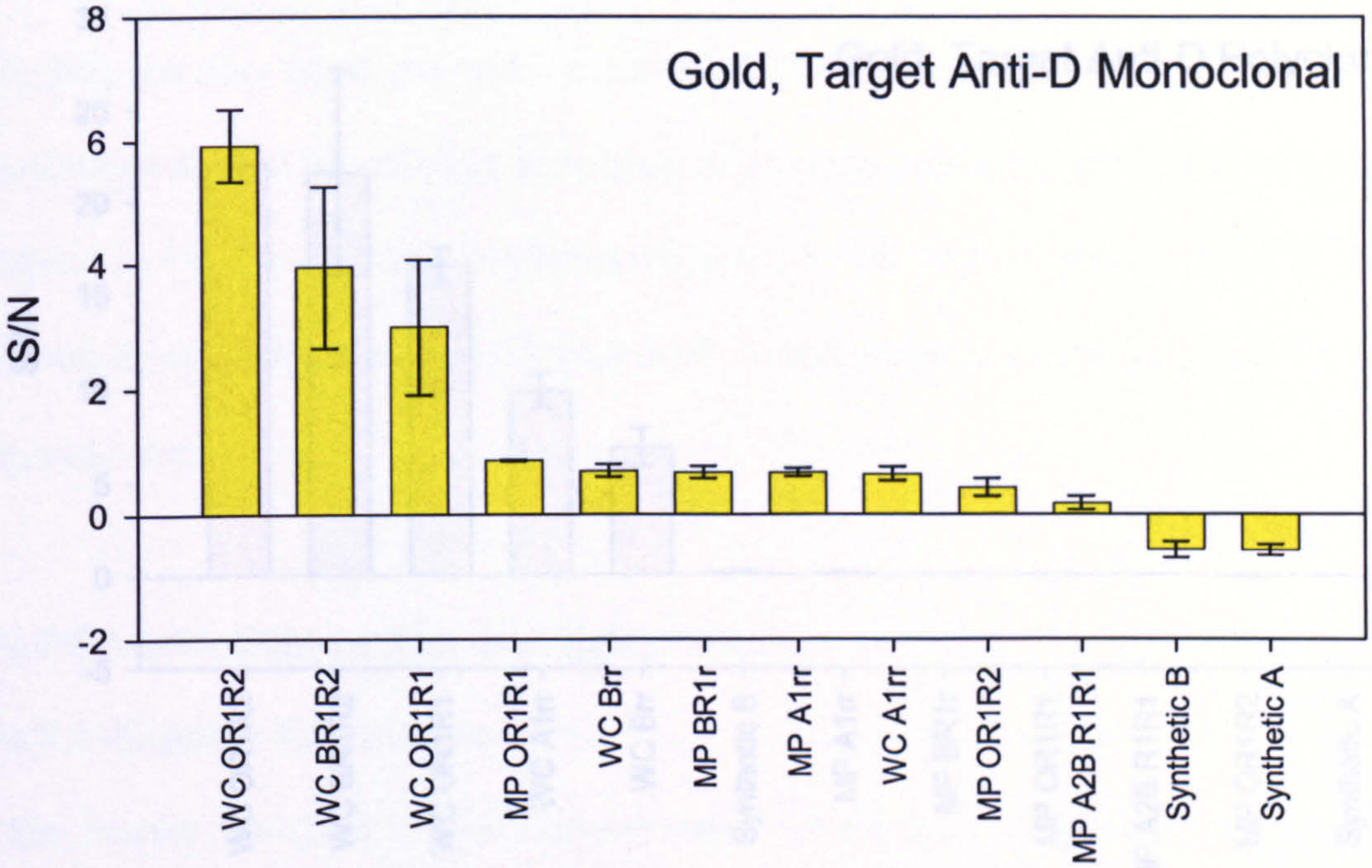



**Figure 6.8b**. Results of BioGold and SuperProtein slides against target anti-B. Slide type detailed in figure, slide reps 1, pins 900 $\mu$m, probes detailed on x-axis, probe reps 3, SPM E; blocker PBS-BSA, target/detection Table 6.8, incubation time 60min, mixing, scanning method C, data processing method B.



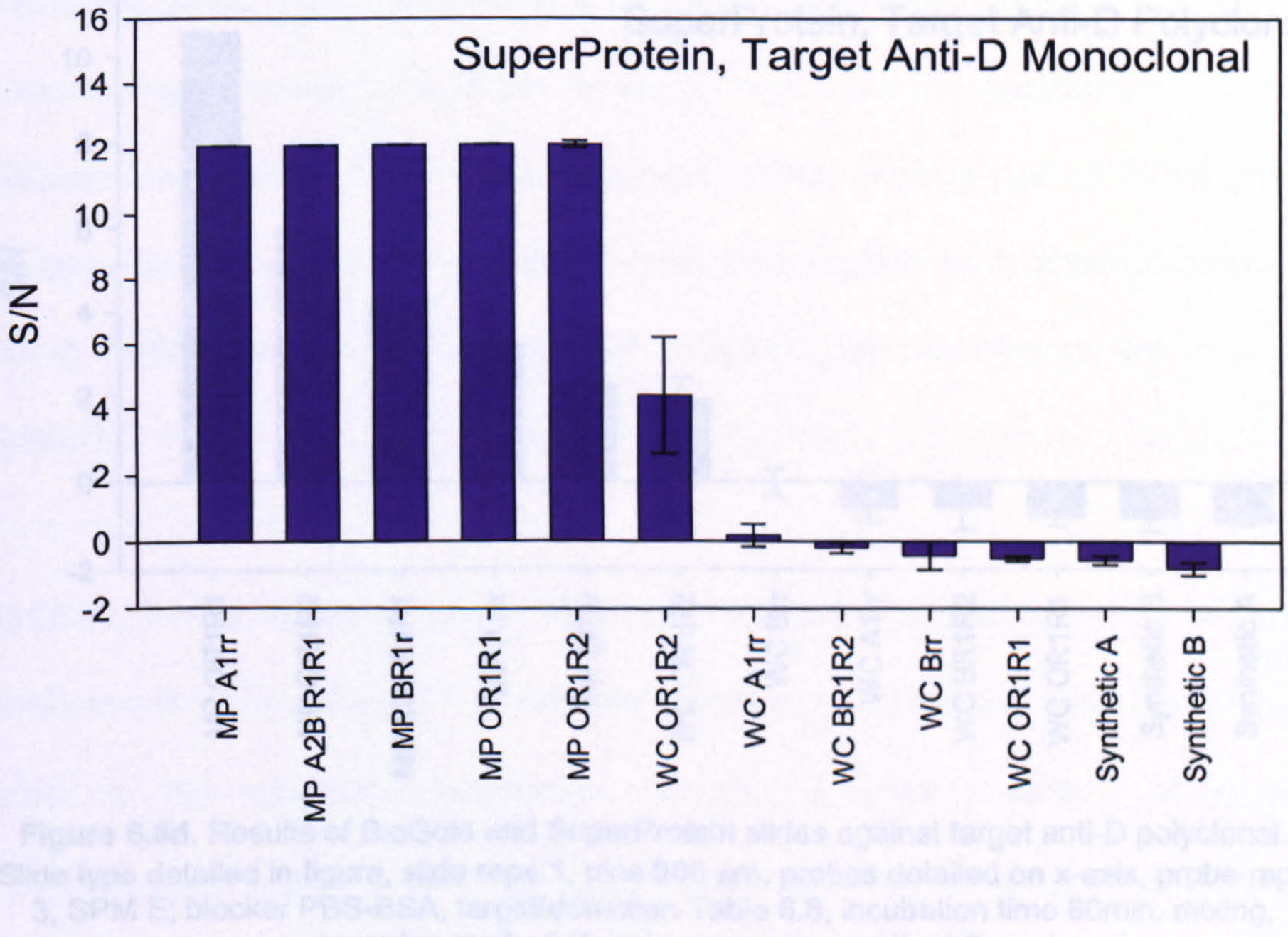




**Figure 6.8c**. Results of BioGold and SuperProtein slides against target anti-D LHM169/80. Slide type detailed in figure, slide reps 1, pins 900 $\mu$m, probes detailed on x-axis, probe reps 3, SPM E; blocker PBS-BSA, target/detection Table 6.8, incubation time 60min, mixing, scanning method C, data processing method B.

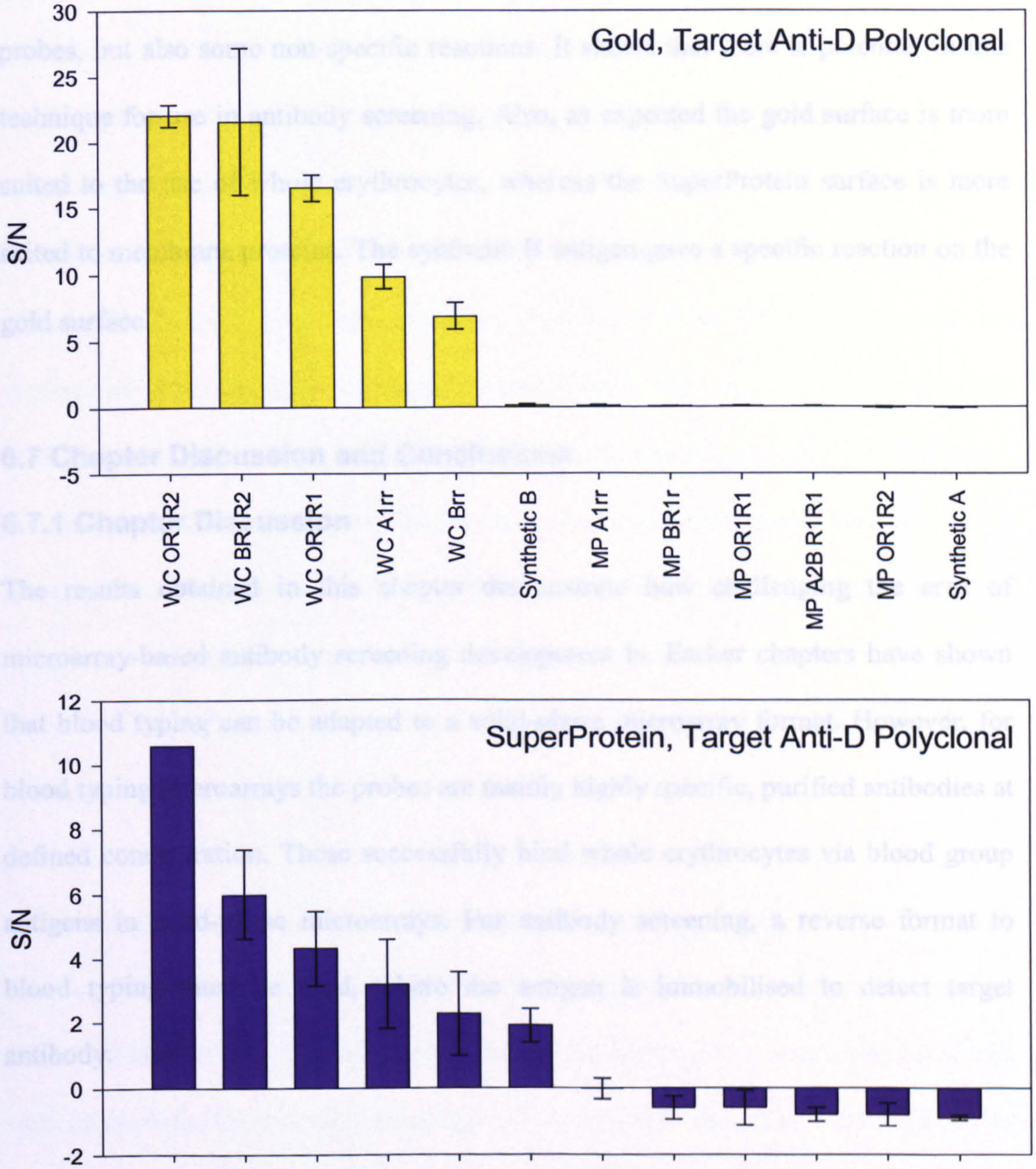



**Figure 6.8d**. Results of BioGold and SuperProtein slides against target anti-D polyclonal. Slide type detailed in figure, slide reps 1, pins 900 $\mu$m, probes detailed on x-axis, probe reps 3, SPM E; blocker PBS-BSA, target/detection Table 6.8, incubation time 60min, mixing, scanning method C, data processing method B.

In conclusion, this experiment has given some encouraging S/N values with specific probes, but also some non-specific reactions. It shows that there is potential in this technique for use in antibody screening. Also, as expected the gold surface is more suited to the use of whole erythrocytes, whereas the SuperProtein surface is more suited to membrane proteins. The synthetic B antigen gave a specific reaction on the gold surface.

## 6.7 Chapter Discussion and Conclusions

### 6.7.1 Chapter Discussion

The results obtained in this chapter demonstrate how challenging the area of microarray-based antibody screening development is. Earlier chapters have shown that blood typing can be adapted to a solid-phase microarray format. However, for blood typing microarrays the probes are mainly highly specific, purified antibodies at defined concentration. These successfully bind whole erythrocytes via blood group antigens in solid-phase microarrays. For antibody screening, a reverse format to blood typing must be used, where the antigen is immobilised to detect target antibody.

Antibody screening probes pose a different problem to the immobilisation of probe antibodies for blood typing. The antigen probes are, themselves, attached in solid-phase to the erythrocyte membrane; therefore a suitable method of antigen immobilisation must be developed. The immobilisation of antigens for use in microarrays has been demonstrated in many publications, but the preparation of erythrocyte microarrays has not yet been published.

Immobilisation of erythrocytes on microplates has been discussed (Scott, 1991; Tamai and Mazda, 1999), but it is known that the lifespan of a complete whole erythrocyte is unlikely to give the desired stability. The lifespan of an erythrocyte is, on average, 120 days, and at any time a sample will contain cells of all stages of the life cycle. Other formats of the blood group antigens may be more suitable for the purpose. If blood group antigens are available from a source other than the erythrocyte, this may have benefits to the blood bank and blood transfusion services as the blood can be used for transfusion. Also, non-erythrocyte derived blood group antigens may offer a more consistent source of antigen and be more cost effective to manufacture. Existing methods for the immobilisation and use of blood group antigens in microplates have used erythrocyte membranes, or 'ghosts'. If these were to be used in a microarray format, another advantage would be a reduction in the volume required.

Immucor patented a method for the immobilisation and drying of cells onto solid supports (Sinor and Eatz, 1991) for use in solid-phase immunoassays. The method described involves the immobilisation of cells by electrostatic charge and the *in situ* lysis of the cells. Once immobilised, the cells are fixed or dried by the use of a drying solution, which draws moisture out of the cells. This method has been successfully used as part of an antibody screening method for many years, and could possibly be adapted for use in microarrays.

Another significant point in the development of an antibody screening method is the non-specific binding introduced when using serum or plasma samples. In the

Immucor system, the target sample is diluted up to 1 in 4 prior to use. In other microarray system publications, the serum samples have been diluted prior to use. Haab *et al.* (2001) stated that using their system, a total protein concentration of less than 1 mg/ml should be used for optimal performance, otherwise NSB of the target protein was evident. In existing donor antibody screening methods using the Olympus system, the plasma is diluted 1 in 6 before testing (G. Glassford, personal communication).

In contrast to the use of purified antibodies, the use of materials such as membrane fragments may result in higher background. It is likely that this is due to many more structures and molecules being exposed on the fragments, causing non-specific binding. Much non-specific binding was evident in these experiments, particularly when using polyclonal material of human source. In the experiment in section 6.3, the NSB may have been due to the presence of other alloantibodies (such as anti-I), which may be only detectable at room temperature. Group O individuals usually produce a mixture of IgM and IgG anti-A and anti-B, but only anti-human IgG was used as the detection molecule. It may have been more appropriate to use anti-human IgM, or a blend of anti-human IgM and IgG, which was not available at the time of the experiment. However, the unwanted reactions of the group O serum with all erythrocytes may be eliminated if the assay was performed at 37 °C. The facility to perform this for microarray slides was not available during the experiment.

Prior to their patent covering cell immobilisation, Immucor patented (Sinor *et al.*, 1989) a method of solid-phase indicator cell preparation. These cells were described

as a means of detecting IgG antibody binding where the secondary binding and subsequent layer of the indicator cells meant a positive reaction. This overcomes the problems of non-specific interaction demonstrated by other detection methods. This method could, theoretically, be used for microarray, but in microarrays fluorescence is most commonly used for detection. If the cells were fluorescently labelled and sensitised with anti-IgG they would bind to the array if IgG antibody was present and give a detectable fluorescent signal. Care would be required to ensure interpretation of results only from the antibody screening probes. Other probes on the microarray could be IgG or anti-IgG and would therefore bind the indicator cells non-specifically. If using this method, the indicator cells could interfere with other tests on the microarray. If desired, this detection method may require the use of a separate chamber on the array. Immucor have used the indicator cell technology described above for the detection of antibodies to platelets, leucocytes, HBsAg, HTLV-III and CMV. For these tests, cells expressing the antigen of interest must be immobilised. The patent covers any solid-support, and therefore also covers microarrays.

The use of solid-phase techniques for serological assays means that it is immune adherence of antibody rather than haemagglutination that is used for detection. Although haemagglutination as an end-point may be automated, it can be somewhat subjective if read manually as individuals may read tests by different techniques. Immune binding of a fluorescent antibody to determine interaction is a far more objective and quantitative end-point than detection of an erythrocyte monolayer or haemagglutination as the result would be interpreted only from a fluorescent value.

In this chapter, three different types of probe have been investigated; whole erythrocytes, erythrocyte membrane (protein) fragments and synthetic carbohydrate-BSA conjugated blood group antigens. These probes were examined for use on different slide surfaces. It is clear that the slide surfaces are suited to different probe immobilisation, likely due to suitable surface-probe interactions. As discussed, whole erythrocytes have a limited life-span, a problem that may be overcome by the use of erythrocyte membranes, cell fragments or synthetic antigens. Other options not explored in this project are the use of peptides or recombinant antigens.

Gold slides were shown to be successful in the immobilisation of whole erythrocytes. This would be an ideal surface on which to develop an antibody screening microarray as blood typing has been demonstrated on this surface in this project. This would facilitate the optimisation and multiplexing of both types of assay, which would be compatible for one comprehensive microarray. The use of probes other than whole cells, were not as successful on gold slides. This is likely due to the properties of the gold surface and the surface plasmon waves that exist on this type of slide (see Campbell *et al.*, Appendix 4). The gold causes quenching of fluorescent signals close to the surface, and detection of fluorescence is therefore distance dependent to the surface. This is one possible explanation for the results from whole cells on gold slides.

However, the stability of whole cells attached to a solid-phase is unlikely to be suitable for such a test system where shelf life of product would ideally be months rather than weeks. Therefore other slide surface coatings were investigated for their

retention of erythrocyte membrane fragments. Slides that demonstrated highest binding of fragments tended to bind by hydrophobic interactions. The method for isolation of the membrane fragments would require standardisation to ensure that all material was at similar concentration. Orientation of the fragments is also random, but occur through hydrophobic interactions. Intrinsic membrane proteins consist of two hydrophilic and one hydrophobic domain (reviewed in Schenkel-Brunner, 2000). The hydrophobic domain is integrated in the lipid bilayer. When used for antibody screening purposes, the membrane fragments, in general, showed significant levels of binding to target antibody, which could be deemed mainly specific. Other methods for attachment of erythrocytes have been developed, (Sinor *et al.*, 1989; Scott, 1991; Tamai and Mazda, 1999) and could be applied to microarrays.

In section 6.3, the monoclonal anti-D used was an IgG molecule, whereas the anti-A and anti-B were IgM. This reflects the presence of these antibody specificities in nature, where circulating anti-A and anti-B antibodies are mostly IgM and alloanti-D is usually IgG. The potency of the polyclonal human group O serum against both group $A_1$ and B cells was 1/32, and the potency of the human anti-D serum was 1/256. The latter had not been adsorbed to remove unwanted antibodies, such as anti-A and anti-B, therefore may have been expected to react with all the erythrocytes, perhaps to different degrees, but it did not react significantly with any. It may be that the dilution (1/10) may have diluted out the antibody to an undetectable level, and as discussed previously the dilution of plasma/serum samples requires further investigation. To ensure the system is capable of weak antibody detection, panels of weak antibodies of clinical significance should be prepared for validation purposes.

It is clear that the area of microarray erythrocyte antibody screening requires much development. Due to the huge potential in this area, Alba Bioscience was awarded funding from Scottish Enterprise for the development of this project area over the next two years.

Apart from suitable surface and probes for antibody screening, other microarray options are applicable to this development. Microarray design options have been previously discussed (in Chapter 1) and applied (in Chapters 3 and 4). The main design options for antibody screening, which are not discussed here but would form the next step of this research and development area, would be as follows:

**Microarray fabrication**. Are solid pins the best option or could other pin types or deposition methods be more suitable?

**Blocking**. Different types of probes and surfaces require the optimisation of blocking steps to minimise NSB. The use of membrane fragments demonstrated the need for improved blocking to reduce non-specific interactions, perhaps caused by the presence of more antigens on the fragments.

**Reaction conditions**. Conditions for the reaction to take place require optimisation of temperature, incubation time, plasma/serum dilutions and reaction volumes.

**Washing procedures.** Procedures have to be optimised to ensure delicate immobilised probes or targets are not removed from the surface, while ensuring the elimination of unbound materials.

**Detection methods.** The most suitable detection method would detect all clinically significant antibodies bound to the microarray. This should be compatible with all probes on the array, and not give indication of a positive reaction by binding directly to a probe. Different coloured fluorophores could be an option. Ideally all detection molecules would be added at one time, for both the blood typing and alloantibody screening. Consideration must also be taken of the type of antibody target. The use of only anti-IgG for detection would fail to detect IgM antibodies. Attention should be paid to the selection of the optimal detection mixture so as not to miss clinically significant antibodies, yet not make the test too sensitive that it detects clinically insignificant antibodies or give false positives. Detection of such clinically insignificant antibodies would mean time consuming further testing in a routine situation. Although some success was seen in this work, the detection levels must be greatly increased, as currently they are not satisfactory for routine use in antibody screening or for reverse ABO typing.

### 6.7.2 Chapter Conclusions

The work of this chapter has demonstrated the following in response to the chapter aims:

- *Investigation of different slide surface chemistries for the immobilisation of novel blood group antigens.*

  Many different slide surfaces were used and those that showed most potential were investigated further. The surfaces selected were either gold surfaced or used hydrophobic interactions to immobilise antigen.

- *Evaluation of blood group antigens in a variety of forms for their use in antibody screening – whole erythrocytes, erythrocyte membrane (protein) fragments and synthetic antigens.*

  All three types of antigen were investigated and each showed specific reactions or more suitable reactions on different slide surfaces, showing that both the antigen format and slide surface chemistry requires careful selection and much development work. Specific reaction patterns were obtained with whole cells and synthetic B antigen on a variety of surfaces, but especially BioGold. Probe membrane fragments gave some specific reactions when using slide surfaces such as P4VP, ZetaGrip and SuperProtein slides. Generally, higher S/N values were found when the targets were monoclonals, probably due to the purity and potency of these materials.

To conclude this thesis, Chapter 7 reviews all the achievements of this work and discusses the implications and future that this study has introduced.

# CHAPTER 7

# DISCUSSION

# 7. DISCUSSION

## 7.1 Achievements of the Project

This thesis has described a novel system to study multiple interactions of blood group antigens and antibodies in a microarray platform, and the exploitation of the results for potential diagnostic use of the system. Overall, this thesis provides a strong basis for further development of a comprehensive microarray based multi-parameter blood group serology diagnostic platform.

Initial experiments focused on multiple parameters affecting the immobilisation and functionality of monoclonal and polyclonal antibodies and serum proteins on various microarray surfaces. Extensive evaluation of parameters important to the preparation and use of protein microarrays has been carried out. The microarray performance was improved during these evaluations, looking at blocking, reaction volume, mixing and time, and process improvements applied to establish an efficient and effective procedure. Selected microarray surfaces and optimised spotting and reaction conditions were then applied to interaction studies using erythrocytes and blood group antibodies.

This thesis provides the first comprehensive study of non-agglutination blood group determination in an open-plan microarray format. Both direct and indirect labelling detection methods were investigated to find both a suitable method of detection for future use and a method that could be used for the development of the system. The optimisation of antibody immobilisation and interaction was further developed by adjustment of spotting buffers, the effect of pH clearly different for each antibody.

This demonstrated that each probe antibody must be optimised individually to give optimal performance. Using the developed microarray methods, both carbohydrate (ABO blood group system) and protein (Rhesus D, c, E and K) blood group antigenic determinants were successfully typed in a simultaneous multi-parameter microarray. The detection of erythrocytes sensitised with IgG was also successfully investigated simultaneously using the microarray approach.

In order to verify performance of blood typing antibodies and quantitatively characterise interactions, the technology of surface plasmon resonance was used as an alternative solid-phase system. This work gave detailed information on the interactions between an antibody to blood group A and erythrocytes of different specificity. Also, the immobilisation of synthetic carbohydrate antigen to a Biacore chip permitted the quantitative determination of the reactivity of monoclonal antibodies in a reverse typing format.

Preliminary work was carried out to extend the use of the microarray platform to include a reverse assay, an erythrocyte antibody screen. For erythrocyte antibody screening purposes, synthetic antigens, whole erythrocytes and erythrocyte membrane protein fragments were used in a microarray format. In this thesis, the different forms of the antigens were used for antibody screening using novel surfaces and subsequently defining the parameters for this purpose. Specific reaction patterns were obtained in many cases. However, the higher level of non-specific interaction and differences in data obtained on different slide surfaces does require substantial further development.

The work of this thesis lays the foundations for further development of microarray based blood group serology. Multi-parameter testing has been performed which helps foresee the potential of this test format for other diagnostic purposes, and also to extend this work to develop a comprehensive blood testing microarray chip. The considerations involved in a comprehensive and automated testing platform are discussed below.

For the first time, blood grouping has been performed in a miniaturised multiplexed, open-plan format. Other blood testing systems differ in that they compartmentalise each probe-target interaction. In the microarray developed in this project, multiple probes could be used to probe a sample for multiple parameters, which are detected in simultaneous analysis. The open-plan microarray gave no apparent problems with cross-contamination of probes, which may be of concern to some when using such a format. Results were confirmed using the technology of surface plasmon resonance, which studied the interactions individually in real-time and gave precise comparisons between probe antibodies and interactions with different cell types. It also gave the basis for the use of synthetic antigens that were subsequently used in antibody screening.

## 7.2 Potential Advantages of a Microarray Platform

Multiplexing is a clear advantage that microarray technology possesses over other existing serological technologies. In the open-plan slide format, the number of probes may be limited only by their availability. The inclusion of new probes for new tests has only a small incremental cost on the preparation of the array, however reagent

costs must be considered for any probe. The microarrays used in this project contained 144 probe spots on average. Even including test replicates on the array, this may be enough to accommodate all current mandatory blood testing. Smaller pins are available and these would allow microarray fabrication to include hundreds of probes. The technology exists in DNA microarraying to fabricate microarrays with many thousands of probe spots. This multiplexing feature is also useful when new tests evolve or are required. Rather than requiring another testing system or machine in the testing laboratory, additional probes could be added to the pre-existing format and be interpreted simultaneously.

When dealing with human blood and transfusion, new tests are currently being developed (e.g. Malarial tests, West Nile Virus), or may be required at any time in the future. The speed at which testing is introduced in a clinical environment can be critical to health authorities, who are under pressure to ensure best practice at all times. Although each new test would require extensive validation before it was routinely used, the technology to apply it to every blood sample is possible using microarrays.

Miniaturisation has two main advantages. 1) A smaller test system such as a microarray can accommodate multiple tests, and 2) when used in an open-plan format it leads to reduced usage of both sample reagents and of human blood samples. This also means that the testing system itself can be made smaller and less space is required.

The amalgamation of all tests on one platform has many cost implications. As mentioned above, a smaller testing system means less space is required for multiple test platforms. This may also mean a reduced cost in running one system as opposed to multiple machines, where rental, reagent and maintenance costs are all to be considered. An obvious effect on costs will be staff requirement. If a fully automated system can run all mandatory and desired tests on one platform, then fewer staff are required to run and interpret the results. There is, of course, no substitute for human experience and expertise, but a well-designed system should minimise user input and may lead to a reduction in the specialist skills required in the workplace.

Interpretation of traditional haemagglutination assays relies on the visible clumping of the cells as an end-point. This reaction can be read by programmed automation, which interprets a photograph of the reaction, or uses spectrophotometric-based analysis. The use of fluorescence (or other available detection methods) in microarrays gives a numerical value, which allows for ease of interpretation, and a clear positive or negative reaction can be obtained with well-developed and validated cut-off points for each reaction.

If a fully automated microarray technology testing platform were to reach the market, it would therefore have some clear advantages over other testing systems involving antibody-antigen interactions. A comparison table is presented as **Table 7.1.**

Although this project has demonstrated that microarray technology can be adapted for diagnostic purposes, microarrays also have immense potential for research use.

**Table 7.1.** Parameters of different blood grouping techniques (table used in Robb *et al.*, accepted to Transfusion Medicine, 2005, see Appendix 4).

| Test method | Antibody probe volume per data point ($\mu$l) | Sample volume per no. of probes ($\mu$l) | Readout method | Throughput: No. of samples (data points) per run | Throughput: No. of samples (data points) in 6 hours |
|---|---|---|---|---|---|
| Manual tube | 40 per test | 40 to 1 | Haemagglutination | 50 (350) | 300 (2100) |
| Microplate | 25 per well | 25 to 1 | Haemagglutination | 240 (2880) | 1440 (17280) |
| Column agglutination technology | 25 per microtube | 50 to 1 | Haemagglutination | 70 (420) | 420 (2520) |
| Surface plasmon resonance | 70 per chip | 70 to 1 | Surface plasmon resonance | 1 (1) | 24 (24) |
| Open-plan protein microarray | 0.02 per spot (if using 700 $\mu$m pins) | 25-450 to n x 10 to n x 100 | Fluorescence | n x 10 (n x 1000) | n x 100 (n x 10000) |

## 7.3 Challenges to the Future of Microarray Technology Testing

Depending on the purpose, there are alternative platforms on which biological interactions can be studied and which may compete with the protein microarray platform. Some of these options are discussed below.

The surface plasmon resonance (SPR) format offers useful information, and allows studies to be performed in real-time, which is something that microarrays currently do not offer. It also has the advantage that labelling of samples is not required due to the method of detection. But real-time studies are not a requirement of diagnostic systems. To use the definition of Ekins (1989), blood typing is an analytical assay, looking for structural specifics, as opposed to a comparative or functionally specific assay. A blood typing assay seeks a yes or no answer i.e. is an antigen or antibody present or not. To an extent a measure of quantity is not required, unless a level of antibody present in a sample is desired. The fact that a marker is present can be enough to diagnose and provide the desired care.

SPR technology can use a variety of immobilisation methods to capture ligand on the sensor surface. Given the problems associated with RhD antigen isolation, especially their conformational requirement to be inserted into the cell membrane for support, then the technology of liposomes could be used to immobilise membrane proteins. Liposomes work by accommodating proteins and allowing them to take on their natural state in the membrane (Biacore.com). If isolated RhD proteins were available this may be one system that could be used to study the interactions with antibodies. Another advantage of the SPR format is the regeneration and re-use of the chips,

allowing for more economical analysis. The covalent attachment of probes means that it is possible to remove target particles and re-use the probe surface. The re-use of microarray chips is possible, although the costs involved would have to be lower than for disposable chips.

NAT testing, or DNA genotyping can now be performed to determine many of the main blood groups, and can be performed to determine the pathogen status of individuals for some diseases. The most recent advances in blood group genotyping were discussed in Chapter 1, where it was also mentioned why, for the foreseeable future, antibody-antigen interactions would remain a critical part of testing. DNA genotyping cannot determine immune antibodies produced by an individual, and unless patients receive identical blood to their own it is likely that antibodies will still be made and require detection to provide compatible blood. It may at some time, however, be beneficial to include traditional antibody-antigen immunohaematology and DNA testing on one microarray. The use of a combined technology chip using microfluidics channels to separate samples was considered by Petrik (2001), and depending on whether antibodies and antigens to all desired targets are available, this type of chip may be the best option. Chiron and Roche have both recently launched new automated NAT systems, demonstrating that the technology to automate such testing is already available.

The Ciphergen system (ciphergen.com) offers affinity capture of probes and can isolate target molecules directly from patient samples. This system allows the verification of sample identity from analysis of the bound molecules, which although

useful, is not necessary for blood testing, as it would be unable to distinguish IgG antibodies from each other, or erythrocytes. The technology also has cost implications and throughput is limited.

The Luminex (TX, USA) 100™ system (luminex.com) uses a suspension array where probes are captured onto beads, which are manufactured with a distinctive proportion of red and orange fluorescent dyes (Carson and Vignali, 1999). A reporter dye is used for detection of bound target and results are read using flow cytometric analysis, which identifies each bead set using one red detection laser and the quantity of target bound using another green detection laser. Although this system has been shown useful in cytokine detection, it is yet to be proven compatible when using cell targets. Likewise, the Illumina BeadChip system (San Diego, USA) (illumina.com), which uses optic fibres bundles and beads to prepare interaction arrays, had not yet been shown capable of dealing with large cells such as erythrocytes.

As many probes can be included on an array, it should be noted that even with around 144 spots per array as in this project, the data generation is large. With even more probes the data generation will increase and data interpretation and presentation would be more difficult. Failure to find adequate data analysis software could be a risk associated with this technology. Another consideration that a small error rate applied across a large data set can give a large number of inaccuracies. For example, if a typical system has 99.9 % accuracy, with 10,000 probes it would give approximately 10 false results. Therefore, the inclusion of replicate probes should reduce imprecision and increase accuracy.

When considering if any technology will challenge the potential of microarrays in the diagnostic field, the answer seems clearly that no other available format could offer the same advantages. As discussed, alternative systems can offer some individual advantages, but some may not be required and may make analysis over complicated. Disadvantages to microarray technology are limited, but it may be that the potential advantages are limited by their nature. These issues must be overcome during the development of a robust system. Issues such as cross reactivity and detection conflicts are further discussed, but it may be that detection is difficult due to cross-reactivity, which may arise with detection of multiple interactions. Any added sensitivity of microarrays may also increase detection of clinically insignificant antibodies.

## 7.4 Is Microarray Technology the Future of Blood Testing?

To achieve the second generation of microarray systems, automated flow-through systems to take samples from start to finish will be required, along with powerful data-processing software; fully integrated systems that combine all components into one single unit. Chapter 1 described how Blohm and Guiseppe-Elie (2001) reported that at least five components are required for a successful microarray assay system. These are the chip/slide on which to build a 'microarray', a device to fabricate the microarrays, a system or method for hybridisation (sensitisation/incubation), and a scanner to read the microarrays and software to process and analyse the results. Equipment for the preparation of samples would be in addition to this. For a new automated microarray test format, all of these components would be required to work as one integrated system. However, it is most likely that the microarrays would be

prepared and supplied ready-for-use by a manufacturer, and only the test performed in test laboratories.

As well as factors such as sensitivity, specificity, costs, speed and the benefits of miniaturisation, the level of automation available for such a test system must be considered. The level of automation can indeed offer benefits to the aforementioned factors. While automation was not evaluated as part of this thesis, it is considered now as it impacts on blood testing microarrays reaching the market.

An automated test system must be capable of positive sample identification, sample processing, sample-chip interaction, processing steps and data interpretation. Both high-throughput systems and point-of-care devices should be considered with a level of automation. Also relevant is the level of user input and level of training required to interpret results. It is possible that taking testing out of a central laboratory to the bedside may result in false interpretation when read by inadequately trained staff, and this would require risk analysis. The support software must offer sophisticated, robust computer programs and testing algorithms to deal with large data set generation.

Along with the consideration of user-friendliness is the issue of controls and calibration. The highly regulated area of blood testing requires the use of valid controls and calibration testing. The subject of controls for such a system is complex, and is discussed in section 7.5.

It is likely that the combination of science and technology required to develop such a system would require the collaboration of many disciplines. SNBTS scientists are currently investigating all aspects of a future blood testing microarray. This thesis has demonstrated that blood typing by microarray is a realistic option, and work has begun on antibody screening. Pathogen antigen arrays have been performed (Ewart *et al.*, 2001; Bacarese-Hamilton and Crisanti, 2002) and are being actively performed by our group. The preliminary results from our Proof of Concept group have shown that blood typing and pathogen testing can be combined in one microarray assay. It may be that pathogen detection will still require an amplification step to allow a suitable level of detection, and this is likely to involve target or signal amplification. The sensitivity of a blood-testing chip is critical. The test system must be of suitable sensitivity to detect clinically significant targets. To offer benefits over existing test systems, a new system must give equal or increased detection of pathogen targets. This may not be achievable without amplification methods. Reverse transcriptase polymerase chain reaction testing or rolling circle amplification are two options to increase the sensitivity of detection. This has implications with regards to the format of a comprehensive blood testing microarray chip. Ideally there would be one reaction chamber into which whole blood (with or without dilution) would be added. If the different probes are directed towards different type targets then there is a possibility that microfluidics could be employed to perform multiple reaction steps within one chip.

Microarray technology could evolve into point of care testing, where testing is performed in the ward, clinic, or home or close to the patient bedside. The

miniaturisation aspect of microarrays would appear to lend itself to this area, where there has been talk of gross development increases over several years.

While expansion of the number of probes is an advantageous factor, care should be taken not to include unnecessary probes, giving the laboratory more information than is required and overcomplicating the process. Clinical significance must be the primary parameter. One consideration, however, is the use of a single chip for all purposes, and only the software licensed to un-lock the results to only those tests required (similar to Randox, Ireland). This method of microarray preparation would aid manufacture and the subsequent quality control testing of batches of microarray slides.

## 7.5 Development to Market Place

To take the described technology from the development stage to the market will require much work. Some of the remaining development is discussed in section 7.6, but discussed here are some of the regulatory and practical issues, as well as marketing considerations, which must be considered before this new technology can reach the blood testing market.

As discussed in Chapter 1, there are many quality system requirements that govern the area of blood testing diagnostics. In order to comply with the European Union *in vitro* Diagnostic Medical Devices Directive (IVD Directive (98/79/EC), the following stages of development must be performed:

- risk analysis of the product and its possible risks to the health of manufacturing staff, users and patients
- performance evaluation of the product in compliance with recommended guidelines
- validation/verification of all procedures involved in its manufacture and use
- extensive stability testing of all components of the test system and especially biological components
- full technical documentation detailing all materials used in the manufacture; and detailed accounts of all design stages.

All stages of the development should comply with the appropriate regulations by adhering to standard operating procedures and product specifications. Voak (1999b) discusses six critical tests for the introduction of a new technology for red cell agglutination. The described tests include tests for sensitivity, replication, titration tests, clinical trials and detection of weak samples/antibodies. All of these things have to be considered within development stages before taking this product closer to the market place. During the development stages, logistics for manufacture scale-up must be reviewed. While the fabrication of microarrays is possible for the production of small numbers of slides, full production batches would most likely consist of thousands of slides. Quality control testing procedures must be put in place prior to manufacture to ensure post preparation batch verification.

For a comprehensive testing system, the provision of suitable controls would be of concern to the manufacturer and the user. Currently there are regulations for all mandatory testing controls, where known positives and negatives are employed for

each single probe assay. Acceptable controls for a multi-parameter test would require careful consideration as there would be cost implications if many control microarrays were used with each sample batch. It is probable that controls would be supplied by an appropriate manufacturer as this may involve the preparation of multi-target control spiked samples. For gene expression data, there are guidelines for standard information formats. These guidelines are known as 'minimum information about a microarray experiment' (MIAME) (Brazma *et al.*, 2001). The use of MIAME guidelines means that users can share data and have comparable data between different locations. This may be beneficial in the future, so that all clinicians may have access to information about all available donor characteristics. Although an automated blood typing system should analyse and interpret results, MIAME could be used as a reference point from which to develop a data analysis system.

For the remaining development work to take place and then to consider a movement from the research laboratory to the market place is likely to take several more years. During this time, it is likely that many companies will be investigating new methods for blood testing and there will be competition. Detailed development, marketing and commercialisation strategies will be required to take this promising technology from the development stage.

## 7.6 Future Work Arising From This Thesis

This thesis has provided a strong basis for the future development of microarray based multi-parameter blood group serology diagnostic platforms for routine, high-

throughput use. The following areas of development should be considered in more detail.

The system should be validated with further erythrocyte samples to test the system and to ensure its capability of blood grouping. For example, further $A_x$ cells and weak D cells should be evaluated, as well as cord blood samples with weak antigen expression. Panels of these cells should be collected for use and may be evaluated by use of lectin reagents. These should be evaluated to rigourously test the sensitivity and detection limits of the system. Minimum specifications are detailed in the Commission Decision on common technical specifications for in vitro diagnostic medical devices (2002/364/EC) (European Parliament website). The results must be highly reproducible and precise (Petricoin III *et al.*, 2002). The panel of antibodies should be optimised to ensure detection of rare or weak cell types. For each antibody specificity tested, the Guidelines for the United Kingdom Blood Transfusion Services (U.K. Blood Transfusion Services, 2002) should be followed to ensure that clinically significant antigens are detectable. Once established, the microarray test platform would require validation to ensure compliance with the European Union *in vitro* Diagnostic Medical Devices Directive (IVD Directive (98/79/EC), (European Parliament website) and the Food and Drug Administration (FDA) regulations if it were to be marketed worldwide. The FDA has already anticipated the expansion of microarray technology into all aspects of healthcare, and uses the guiding principle of benefit versus risk analysis (Petricoin III *et al.*, 2002), and recommends the involvement of the relevant regulatory body early in the development process.

An initial stage of microarray erythrocyte antibody screening has been carried out in this project. This area could be further developed to increase specificity and sensitivity. Whole erythrocytes, fragments and synthetic antigens were used, but other forms of blood group antigen could also be spotted onto the microarray and their use explored for this purpose. Alternatives to native antigens were mentioned in Chapter 1 and include the use of peptides and aptamers. These areas are currently being actively researched by the SNBTS. These alternative forms of antigens may offer advantages in terms of manufacture and cost, concentration, specificity and stability.

The stability of both probes and spotted microarrays is an area that requires critical examination and must be performed. An excellent probe in terms of performance on a microarray (sensitivity and specificity) could possibly be rendered useless in terms of stability. The purity of probes may be a factor in the stability of protein probes and the formulation may be of paramount importance. The expense of purified antibodies may be limiting and it is unclear what level of purity is best for stability. The inclusion of stabilising proteins, glycerol (MacBeath and Schreiber, 2000), salts and sugars (Lee and Kim, 2002) may assist long-term stability and requires investigation. The method of storage may also affect stability. Both freezing and lyophilisation should be investigated for long-term storage. Extensive stability testing (European Union *in vitro* Diagnostic Medical Devices Directive (IVD Directive (98/79/EC)) should be carried out, which includes the stability of the probes both prior to spotting and following spotting by performing on-microarray stability. Both accelerated and real-time studies should be performed as well as transport studies of the microarrays,

performed to ensure the product is still functional and meets product specification once it reaches the customer.

During this project, it was always considered that blood typing and antibody screening might be included as part of a comprehensive blood testing microarray. These considerations included the type of surface and that the detection of interaction between each probe and target set must be compatible with other sets on the array. This is relatively simple when considering only blood typing, or only antibody screening, but may be complicated if all mandatory tests were amalgamated on one microarray. For example, many of the probes used in this project were monoclonal anti-human IgG to human blood group antigens. This thesis looked at the use of directly labelled erythrocytes, and secondary (indirect) labelling via another molecule that bound to all cells. Both approaches were successful. However, if the reaction was negative i.e. no erythrocytes were bound to the human IgG probe, and this was on the same open-plan microarray as the antibody screening testing, then false positive reactions would be possible as probe human IgG would bind the detection antibody, anti-human IgG. It is most likely that the detection method for antibody screening will use anti-human IgG and perhaps anti-human IgM reagents. Compatibility may be even more complicated once other testing systems are included and multiple detection reagents are required. One possible method to avoid this problem would be the use of multiple fluorophores that use distinct excitation and emission wavelengths. Using this method, a signal of a certain wavelength would be expected from a certain probe spot when scanned using specific laser settings.

Although not a mandatory test, the Direct Antiglobulin Test (DAT) has been demonstrated in this thesis in the microarray format (Chapter 4). It may be advantageous to include this test on a comprehensive testing format, especially in patient testing scenarios. A positive DAT result can be indicative of many clinical situations including autoimmune disorders. It would be of interest to further investigate this test to determine the effect of cell sensitisation on blood typing, and to determine the sensitivity of the technique in this format. The use of multiple fluorophores could also benefit tests such as to perform differential Indirect Antiglobulin Testing. Some testing laboratories like to differentiate between immune antibody classes in serological testing. An anti-human $IgG_1$ reagent could be labelled with a fluorophore and an anti-human $IgG_3$ with another enabling the serologist a differential reading.

The scanning of unlabelled erythrocytes gave an indication that autofluorescence may be a possible form of detection of cell binding. This could be further investigated to develop optimal scanning settings to detect the fluorescence and ensure the desired level of sensitivity. As erythrocyte binding to a microarray is a visible phenomenon, the use some form of photography or reading through the slides may be an approach worthy of investigation, especially if using multiple detection antibodies for detection of other interactions.

To reduce testing times for blood typing on microarrays, and to increase sensitivity, the use of low ionic strength solutions could be further investigated (this was touched

on in Chapter 4). A reduction in testing times may be an advantage, especially to produce a high-throughput system.

Given the basis that the work presented in this thesis represents, and once these areas discussed are investigated successfully, it could be estimated, given appropriate time and resources, that a blood testing microarray may reach the market in the next five to ten years.

In summary, this work has exploited microarray technology to develop a novel approach for potential use in blood testing. The platform developed clearly has the potential to compete with existing technology in terms of speed, sensitivity, throughput and costs. While there may now be a key focus in the area of commercial development of the test system, there is a great deal of optimisation work to be done, and the benefits of protein microarrays as research and investigative tools should also be investigated for other purposes.

# CHAPTER 8

# REFERENCES

## 8. REFERENCES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zyomyx.com

# APPENDICES

## Appendix 1. Manufacturers and Contact Details

This appendix contains the contact details (or distributors) of manufacturers whose products were used in this thesis.

| COMPANY | ADDRESS |
|---|---|
| Actigen Ltd. | Affitech AS, Gaustadalleen 21, 0349 Oslo, Norway. |
| Affymetrix UK Ltd. | Voyager, Mercury Park, Wycombe Lane, Wooburn Green, High Wycombe U.K. HP10 0HH |
| Alba Bioscience (formerly Diagnostics Scotland) | 21 Ellen's Glen Road, Edinburgh, UK, EH17 7QT |
| Amersham BioSciences UK Ltd. | Amersham Place, Little Chalfont, Buckinghamshire, U.K. HP7 9NA |
| Amersham Pharmacia | As Amersham BioSciences U.K. Ltd. |
| Arrayit | TeleChem International, Inc., ArrayIt® Division 524 East Weddell Drive, Sunnyvale, CA 94089, USA |
| Axon Instruments | Molecular Devices Corporation 1311 Orleans Drive, Sunnyvale, CA 94089. |
| Baxter Healthcare Ltd. | Thetford, Norfolk, U.K. |
| BDH | McQuilkin & Co., 21 Polmadie Avenue, Glasgow, U.K. G5 0BB |
| Becton Dickinson | 21 Between Towns Road, Cowley, Oxford, U.K. OX4 3LY |
| Biacore AB | Rapsgatan 7, SE-574 50 Uppsala, Sweden |
| BioRobotics | Apogent Discoveries, Corporate Headquarters, 22 Friars Drive Hudson, NH 03051 U.S.A. |
| CalbioChem | As Merck Biosciences, U.K. |
| Cecil Instruments Ltd. | Milton Technical Centre, Cambridge, U.K. CB4 4AZ |
| Corning | As BDH |
| Dextra Laboratories | Science and Technology Centre, Earley Gate, Whiteknights Road, Reading, U.K. RG6 6BZ |
| DiaMed AG | 1785 Cressier Sur Morat, Switzerland |
| Eppendorf-Netheler-Hinz GmbH | D-22331 Hamburg, Germany |
| Erie Scientific, U.S.A. | VWR International Ltd Merck House, Poole, U.K. BH15 1DT |
| Filtron (PALL Filtron) | VWR International Ltd. Merck House, Poole, U.K. BH15 1TD |

| | |
|---|---|
| Gene Machines | A1-Biotech UK, Anachem House, Charles Street, Luton, Bedfordshire, U.K. LU2 0EB |
| Genetix Ltd. | Queensway, New Milton, Hampshire, U.K. BH25 5NN |
| GSI Lumonics Inc. | 105 Schneider Road, Kanata, Ontario, Canada |
| ID Bio | Parc E.S.T.E.R. Technopole, 6 Allée Skylab, B.P. 6806, F-87068 LIMOGES Cédex, France |
| Invitrogen | PO Box 2312, 9704 CH Groningen, The Netherlands |
| ISCO Inc. | PO Box 5347, 4700 Superior Street, Lincoln, Nebraska, U.S.A. 65805 |
| Jouan Ltd | Merlin Way, Quarry Hill Road, Ilkeston, Derbyshire, U.K. DE7 4RA |
| Merck Biosciences | Padge Road, Beeston, Nottingham U.K. NG9 2JR |
| Microsoft | Microsoft Ltd, Microsoft Campus, Reading, U.K. RG6 1WG |
| Millipore UK Ltd. | Units 3&5 The Courtyards, Hatters Lane, Watford, U.K., WD18 BYH |
| National Blood Service (Bristol) | Southmead Road, Bristol, U.K. BS10 5ND. |
| Novex | 11040 Roselle Street, San Diego, California, U.S.A. 92121 |
| Nunc | Life Technologies Ltd, 3 Fountain Drive, Inchinnan Business Park, Paisley, U.K. PA4 9RF |
| Packard Bioscience | PerkinElmer Life and Analytical Sciences Via Tiepolo, 24, -20052 Monza (Milano), Italy. |
| Pierce | Perbio Science UK Ltd. Century House, Tattenhall, Cheshire, U.K. CH3 9RJ |
| Premier Brands UK Ltd | PO Box 8, Moreton, Wirral, Merseyside, U.K. CH46 8XF |
| Schleicher & Schuell | VWR International Ltd. Merck House, Poole, U.K. BH15 1TD |
| Scottish Centre For Genomic Technology & Informatics | The University of Edinburgh, The Chancellor's Building College of Medicine,49 Little France Crescent, Edinburgh, U.K. EH16 4SB |
| Serologicals (now Celliance) | Fleming Road, Kirkton Campus, Livingston, U.K., EH54 7BN |
| Sigma-Aldrich Company Ltd. | Fancy Road,Poole, Dorset, U.K. BH12 4QH |

| | |
|---|---|
| Sysmex | Sysmex House, Garamonde Drive, Wymbush<br>Milton Keynes, U.K. MK8 8DF |
| Systat Software | Systat Software U.K. Limited, 23 Vista Centre, 50<br>Salisbury Road, Hounslow, London, U.K. TW4 6JQ |
| The Binding Site | PO Box 4073, Birmingham, U.K. B29 6AT |
| V&P Scientific Inc. | San Diego, CA, U.S.A. |
| Wallac | PerkinElmer Life and Analytical Sciences<br>Via Tiepolo, 24, -20052 Monza (Milano), Italy |
| Watson-Marlow Ltd. | Falmouth, Cornwall TR11 4RU,<br>or via VWR International Ltd. |

## APPENDIX 2. Concanavalin A calculations and testing

To determine the optimal quantity of ConA to label an erythrocyte, the following calculations were performed. At pH 7.0 the molecular weight of ConA was estimated as 76,500 Da per molecule, therefore the number of molecules in one mg was calculated:

$$1 \text{ Da} = 1.657 \times 10^{-24} \text{ g}$$

$$76{,}500 \text{ Da (one molecule of ConA)} = 1.27 \times 10^{-19} \text{ g}$$

$$1 \text{ mg of ConA} = 1/1.27 \times 10^{-16} = 0.8 \times 10^{16} \text{ molecules}$$

Determination of the number of ConA molecules required to give a theoretical number per erythrocyte was calculated assuming the use of a 2 % suspension (estimated to contain $2 \times 10^8$ erythrocytes/ml). The details for ConA required are given in **Table A1.**

To add ConA to give a theoretical number of molecules/erythrocyte:

$$\text{ConA/erythrocyte} = \text{molecules required/molecules per mg}$$

$$\text{So for 100 ConA/erythrocyte} = 2 \times 10^{10} / 0.8 \times 10^{16} \text{ molecules}$$

$$= 0.025 \ \mu\text{g required (so, if using ConA 0.1 mg/ml, 2.5 } \mu\text{l required/ml)}$$

**Table A1.** Detail of the number of ConA molecules to be added to 1 ml of cells to give an amount per erythrocyte, if using cells at $2 \times 10^8$/ml (estimated).

| No. of ConA per erythrocyte | No. of ConA molecules required/1 ml |
|---|---|
| 100 | $2 \times 10^{10}$ |
| 1,000 | $2 \times 10^{11}$ |
| 10,000 | $2 \times 10^{12}$ |
| 100,000 | $2 \times 10^{13}$ |
| 1,000,000 | $2 \times 10^{14}$ |

**Table A2.** Results of FACS analysis on erythrocytes labelled with fluorescent ConA. Number of events 'gated' describes the number of cells (from 10,000 counted) which fall within the allocated level of fluorescence.

| Label & quantity | No. of events Gated | % of total labelled |
|---|---|---|
| Unlabelled | 1 | 0.01 |
| FITC ConA, 100 per cell | 5 | 0.05 |
| FITC ConA, 1K per cell | 26 | 0.26 |
| FITC ConA, 10K per cell | 110 | 1.1 |
| FITC ConA, 100K per cell | 9882 | 98.82 |
| FITC ConA, 1million per cell | 9933 | 99.33 |
| Cy3 ConA, 100 per cell | 5 | 0.05 |
| Cy3 ConA, 1K per cell | 4 | 0.04 |
| Cy3 ConA, 10K per cell | 2 | 0.02 |
| Cy3 ConA, 100K per cell | 49 | 0.49 |
| Cy3 ConA, 1 million per cell | 9442 | 94.42 |

## APPENDIX 3. Optimisation of Epoxy Silane Coated Slide Blocking

This is discussed here as it formed the basis of a change from non-fat milk blocking to BSA blocking for all blood typing microarrays. Epoxy silane slides were optimised using this blocking and subsequently this method was used to block all blood typing microarrays performed on gold-coated slides. This is an appendix as it was not directly related to the work presented in this thesis, but did have some impact on processing.

Epoxy silane coated slides were prepared by the SCGTI. The probes printed were detailed in **Table 4.9.** Four replicates of each probe were printed and 700 $\mu$m pins were used. FITC group B cells were used and were suspended in the appropriate blocking solution. The blocking solutions investigated were as follows at varying concentrations; non-fat milk, bovine serum albumin (BSA), non-hydrolysed casein, hydrolysed casein and PBS. The concentrations are detailed on the result figures.

The results are detailed in **Figure A1.** For the expected negative probes, only data from the highest concentration is presented for clarity. The results show higher S/N values than previously using poly-L-lysine slides and that the non-fat milk, BSA and PBS blocking give the highest specific values. Although the PBS has comparatively low S/N it still give sufficient specific reactions. Those blocked with casein all showed high background and therefore low S/N values. The casein appears to have a detrimental effect on the Ab-Ag interaction. For these reasons non-fat milk, BSA and PBS were selected for further evaluation. The results are presented in **Figure A2.** When using three replicate slides the results are calculated from the combined S/N

values for each spot from the three slides. The 1 % BSA was selected as the blocker for subsequent experiments using epoxy silane slides. This is a more convenient blocker to use than the non-fat milk. Although PBS gave good results it was decided that no blocking might cause NSB at some time.

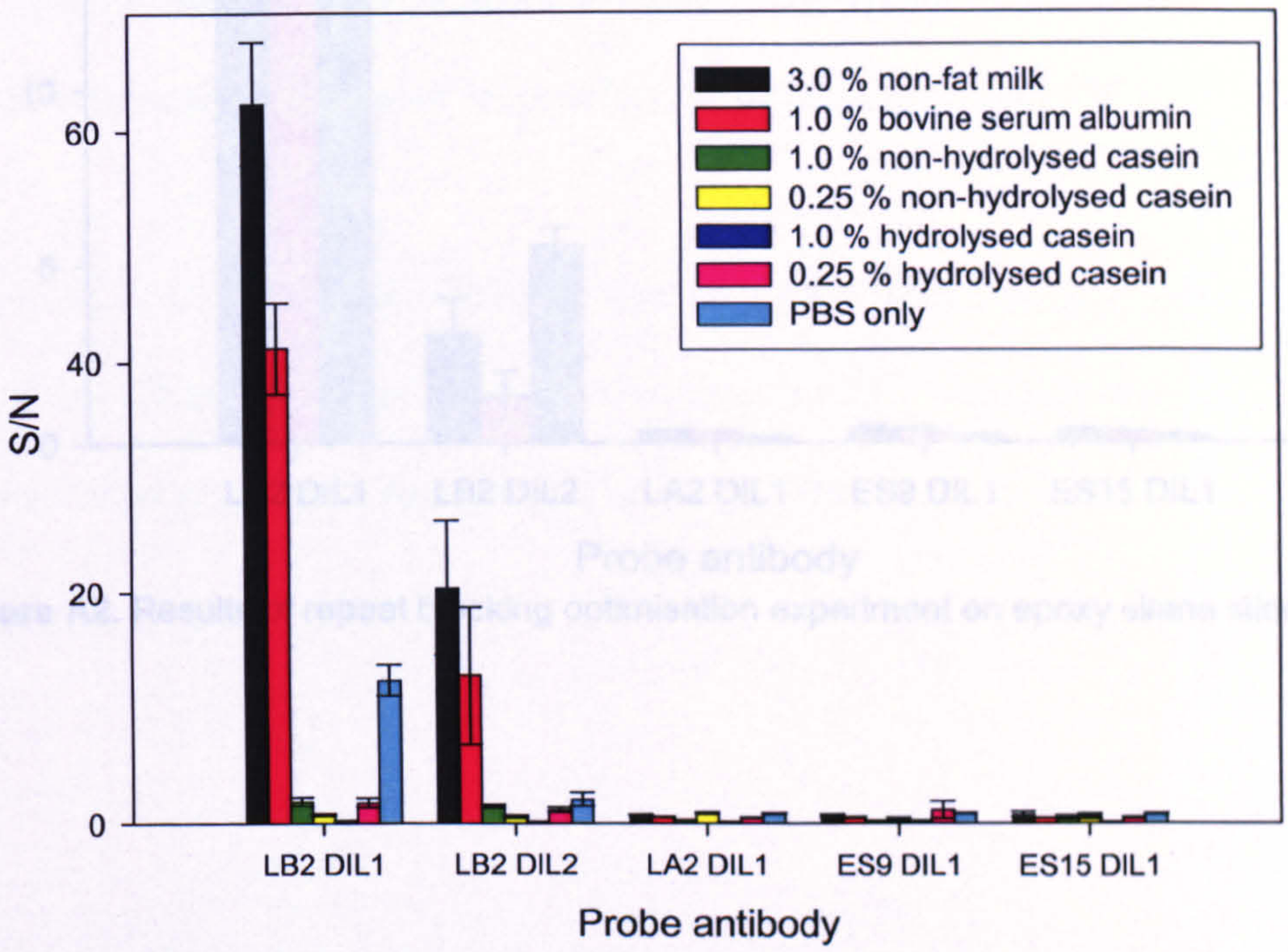


**Figure A1**. Results of initial blocking optimisation experiment on epoxy silane slides.

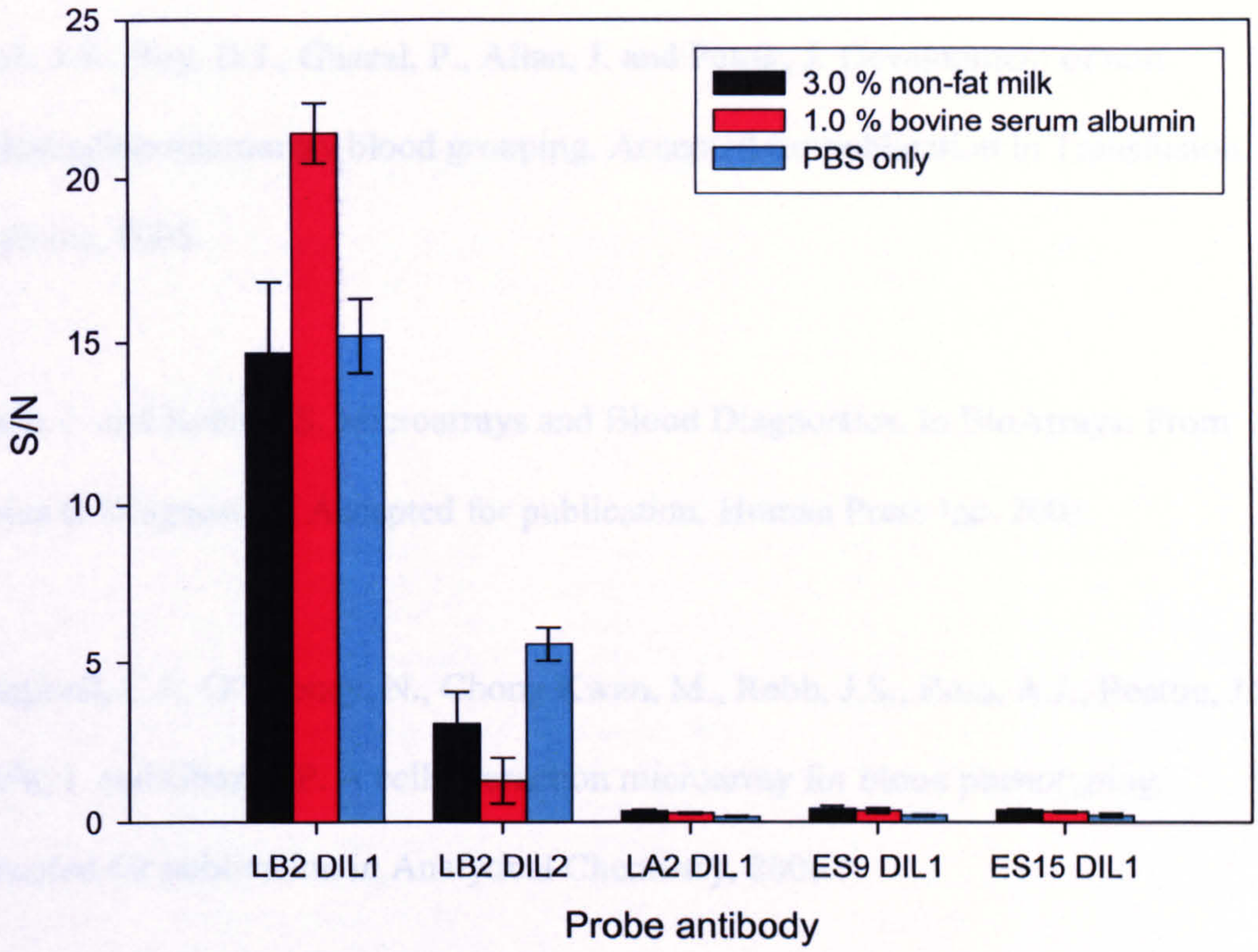


**Figure A2**. Results of repeat blocking optimisation experiment on epoxy silane slides.

## APPENDIX 4. Publications Arising from this Thesis

Robb, J.S., Roy, D.J., Ghazal, P., Allan, J. and Petrik, J. Development of non-agglutination microarray blood grouping. Accepted for publication in Transfusion Medicine, 2005.

Petrik, J. and Robb, J.S. Microarrays and Blood Diagnostics. In BioArrays: From Basics to Diagnostics. Accepted for publication. Human Press Inc. 2005.

Campbell, C.J., O'Looney, N., Chong Kwan, M., Robb, J.S., Ross, A.J., Beattie, J.S., Petrik, J. and Ghazal, P. A cell interaction microarray for blood phenotyping. Accepted for publication in Analytical Chemistry, 2006.

CE: PRR CSE: MB TME 628

*Transfusion Medicine*, 2005, **00**, 1–11 doi: 10.1111/j.1365-3148.2005.00628.x

ORIGINAL ARTICLE

# Development of non-agglutination microarray blood grouping

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2 *Received XX XXXX XXXX; accepted for publication XX XXXX XXXX*

SUMMARY. Microarray technology provides an opportunity to monitor multiple parameters simultaneously. High-throughput applications such as blood donation screening could greatly benefit from performing various tests on a single testing platform. Blood grouping represents one part of the donation testing complementing the screening for blood-borne pathogens. Blood group serology traditionally exploited agglutination as the detection method. In this investigation, we have adapted blood grouping reactions to a solid-phase microarray substrate in a non-agglutination reaction format as an initial step in the development of a combined microarray testing platform. We have investigated immobilization of proprietary antibodies on multiple surfaces and monitored their performance under various reaction conditions. For the first time, highly specific blood grouping has been achieved on a planar microarray using directly labelled erythrocytes or a secondary labelled reagent using fluorescent signal end point readout. We have also complemented microarray data with a label-free, surface plasmon resonance-based Biacore platform data and used the real time quantitative measurement to rank anti-A antibodies according to the strength of reaction with the immobilized synthetic blood group antigen A.

*Key words*: blood grouping, microarrays, protein arrays, solid phase.

Haemagglutination in liquid phase has long been the method of choice for blood group serology. Tubes gradually replaced slide and tile reactions, but ever increasing test numbers have driven the development of new assay formats such as microplate. Solid-phase assays (Beck *et al.*, 1984; Sinor *et al.*, 1985) offered better storage of pre-dispensed dried reagents, higher standardization and easier automation, including operator-independent readout. A microplate format (Wegmann & Smithies, 1966) with immobilized specific antibody became popular for ABO/D grouping, taking advantage of instrumentation already in use for microbiology blood screening. Various parameters affecting the assay, such as the purity and concentration of antibody, pH and ionic strength during the coating procedure and chemical composition of coated surfaces have been investigated in detail (Scott, 1991). The need for repeated microplate centrifugation prompted development of an alternate solid-phase assay – the gel card system containing microtubes with antibodies suspended within a gel (Lapierre *et al.*, 1990; marketed by DiaMed) or 3
glass microbeads (Reis *et al.*, 1993; marketed by Ortho Clinical Diagnostics). This system requires 4
only final centrifugation and no washes, but it is generally more expensive than microplates. Alternative solid-phase, membrane-based dipstick assays (Plapp *et al.*, 1986) are used mostly in bedside blood grouping, as they are not designed for high-throughput applications.

Agglutination techniques on solid phase have been exploited with lesser success in the reverse format to test for clinically relevant alloantibodies against blood group antigens. It proved more difficult to reproducibly immobilize and store erythrocytes or erythrocyte ghosts than immobilized antibodies (Scott, 1991; Knight & de Silva, 1996), and despite various systems being developed (Ramsey *et al.*, 1977; Uthermann & Poschmann, 1990; Beck *et al.*, 5
1991; Rolih *et al.*, 1995), gel cards are still the most

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widely used format. Centrifugal forces may sometime break weak agglutinates leading to the failure to detect some weak antibodies (Phillips *et al.*, 1997).

Microplate format dominates the field of immunoassays in general. Attempts to further miniaturize these assays started as far back as the late 80s, in relation to the development of multianalyte assays and based on a theoretical prediction that they may require much smaller amounts of antibodies than originally thought (Ekins, 1989, 1998). This work has been facilitated by the initially unrelated development of DNA arrays. Aimed at detecting simultaneous expression profiles of multiple genes and driven by large sequencing projects providing the content for expression profiling microarrays, these arrays are produced by contact printing of cDNAs (Schena *et al.*, 1995) or *in situ* lithographic synthesis of oligonucleotides (Fodor *et al.*, 1991). The transition from porous membranes to non-adsorbent silica-based materials and new surface chemistries led to a significant reduction in reaction volumes, increasing the reaction kinetics. While genomic research was the main driver for the development of these techniques, it soon transpired that diagnostics could not only greatly benefit from them but may also become the main application in the future. In blood banking, microarrays may provide improved antibody screening (Voak, 1999), as well as a desirable single platform for performing a variety of tests simultaneously (Petrik, 2001).

The development of protein microarrays lagged behind DNA microarrays mainly due to the more complex patterns of protein interactions compared with those governing re-annealing of complementary nucleic acid strands. The first protein microarray experiments involved interactions of expression libraries immobilized on porous membranes (Bussow *et al.*, 1998; de Wildt *et al.*, 2000) but new, non-porous materials and printing techniques used for DNA microarrays were quickly adopted. [6] MacBeath & Schrieber, 2000) described contact printing of nanolitre amounts of selected protein solutions on treated glass slides resulting in a covalent attachment allowing the analysis of protein–protein and protein–small molecule interactions. Multianalyte antibody–antigen interactions were investigated by Haab *et al.* (2001) on poly-L-lysine-coated slides. Other modifications included the use of polyacrylamide patches and subarrays within microplate wells (Mendoza *et al.*, 1999; Arenkov *et al.*, 2000). Numerous other microarray systems were developed, bead-based or electronic flat arrays, in some cases utilizing microfluidics (Cheng *et al.*, 1998; Michael *et al.*, 1998; Vignali, 2000).

Blood typing has been demonstrated in solid surface systems other than microplate. Quinn *et al.* (1997) described successful ABO grouping using the commercial Biacore platform, which provides useful information on interactions detected by surface plasmon resonance. The Biacore platform in its current configuration is not, however, designed for a cost-effective mass screening with considerable sample throughput such as blood donation screening. Open plan microarrays offer a number of potential advantages for blood typing analysis.

The aim of this study was to develop an array platform for the discrimination of the ABO blood group system antigens using a set of well-defined proprietary antibodies, which are routinely used in current agglutination-based assays. A number of critical factors were considered in the development of arrays. These were definition of microarray substrates and spotting conditions preserving the antibody functionality; definition of reaction conditions and definition of methods for fluorescent detection of bound erythrocytes. In this paper, we show for the first time successful non-agglutination blood typing using an open plane microarray and a fluorescence readout. We have also complemented microarray data using an established analytical Biacore platform based on the label-free surface plasmon resonance detection method. Biacore real time interaction analysis provided further characterization of reagent antibodies on a solid phase and a method of ranking the antibodies using a reverse assay with the immobilized synthetic blood group A antigen.

## MATERIALS AND METHODS

### *Antibodies, antigens and sera*

The antibody probes consisted of a panel of established blood group antibody reagents, well characterized using routine blood group serology agglutination assays. The majority of antibodies are proprietary Scottish National Blood Transfusion Service reagents extensively used in a wide range of tests. A list of antibodies and sera utilized in this study is given in Table 1. Immunoglobulin (Ig)M antibodies were purified either by gel filtration or by affinity chromatography. For gel filtration, a pre-filtered culture supernatant was loaded onto a 500-mL Sephacryl S-300 column connected to UV detector. The biological activity of material in collected fractions was measured by haemagglutination assay. The eluted antibodies were concentrated using appropriate Amicon Ultra centrifugal filter devices (Millipore, UK).

**Table 1.** Antibodies and sera used

| Antibody/serum identification | Antibody specificity | Immunoglobulin class | Source |
|---|---|---|---|
| LA2 | Anti-A | Monoclonal mouse IgM | Alba Bioscience |
| ES9 | Anti-A | Monoclonal mouse IgM | Alba Bioscience |
| LB2 | Anti-B | Monoclonal mouse IgM | Alba Bioscience |
| ES15 | Anti-A(B) | Monoclonal mouse IgM | Alba Bioscience |
| LDM3 | Anti-D | Monoclonal human IgM | Alba Bioscience |
| ESD1 | Anti-D | Monoclonal human IgG | Alba Bioscience |
| LHIVG1 | Anti-HIVgp140 | Mouse IgG (monoclonal) | Alba Bioscience |
| Cy3 anti-human IgG | Anti-human IgG | IgG | Sigma |
| Goat IgG | Not applicable | IgG | Sigma |
| Sheep IgG | Not applicable | IgG | Sigma |
| Rabbit IgG | Not applicable | IgG | Sigma |
| Human serum | Not applicable | Not applicable | SNBTS donor panel |
| Bovine serum | Not applicable | Not applicable | Alba Bioscience |

Ig, Immunoglobulin.

Affinity chromatography was carried out on 2-mercaptopyridine (1-mL HiTrap IgM Purification 7 HP Columns, Amersham Biosciences, UK) according to the manufacturer's instructions.

IgG antibodies were affinity purified on Prosep G or Prosep A (Millipore, UK) according to the manufacturer's protocol.

Antibody functionality was tested using standard serological haemagglutination assays, and antibodies were quantified by enzyme-linked immunosorbant assay (ELISA).

*Labelling of erythrocytes*

Erythrocyte labelling followed the method of Dr D. Pepper (personal communication). Whole blood was collected into 7-mL tubes containing ethylenediaminetetraacetic acid (EDTA). The pH of a 2% suspension of washed erythrocytes in phosphate buffered saline (PBS) pH 7·0 was adjusted to between 8·5 and 9·0 using 1 M NaOH. Ten mililigram per millilitre of fluorescein isothiocyanate (FITC) in dimethyl sulphoxide was added to a final concentration of 0·1 mg $mL^{-1}$ of FITC. The pH was then re-adjusted to between 8·5 and 9·0. The solution was incubated at 37 °C for 2 h then washed five times in PBS pH 7·0 and then suspended in PBS containing 2% bovine serum albumin (BSA). Fluorescence-activated cell sorter (FACS) analysis was performed on labelled erythrocytes using a Becton Dickinson FACScan. FITC labelled erythrocytes were detected in the FL1 (flow channel one) channel and demonstrated that using this FITC labelling method erythrocytes were labelled to over 99% efficiency (data not shown). Erythrocytes to be used in microarrays were suspended in microarray blocking buffer, as detailed in the processing of microarrays section below.

*Preparation of unlabelled erythrocytes*

Whole blood was collected into 7-mL tubes containing EDTA. Erythrocytes were washed at least four times in PBS pH 7·0 and resuspended at the required concentration in PBS. Erythrocytes to be used in the Biacore system were suspended in HEPES buffered saline (HBS) 10 mM HEPES, 0·15M NaCl, 3·4 mM EDTA and pH 7·4 buffer.

*Printing of microarrays*

Antibody or serum proteins to be printed onto nitrocellulose-coated slides (FAST™ slides, Schleicher & Schuell, Germany) were prepared in 1× array buffer 8 (Schleicher & Schuell). Proteins to be printed onto other array substrates were prepared in PBS pH 7·0. Poly-L-lysine slides were prepared in-house, amino silane slides were purchased from Corning, and polyacrylamide (Hydrogel) slides were purchased from Packard Biosciences. All antibody probes were at a concentration of 50 μg $mL^{-1}$, except LA2, which was printed at 200 μg $mL^{-1}$, and serum proteins at 3 mg $mL^{-1}$. All samples for deposition were dispensed into a 384-well source microplate. Slides were printed using a MicroGrid II Arrayer (BioRobotics) using a print head containing 16 solid 9 pins, 700 μm in diameter. At least three replicates of each sample were printed on each slide. Each slide contained typically 144 spots. Following printing, the arrays were air-dried for 1 h.

*Processing of microarrays*

For the development of the antibody-red blood cell (RBC) antigen screening array, we have modified the method described by Haab *et al.* (2001). All arrays, apart from nitrocellulose, were re-hydrated over PBS pH 7·0, then rinsed in PBS pH 7·0 containing 3% non-fat milk or 1% BSA and 0·1% Tween 20 (Sigma). A blocking was carried out in blocking buffer (PBS pH 7·0 containing 3% non-fat milk or 1% BSA for 1 h). A hybridization chamber (Schleicher & Schuell; 450 μL volume) or Lifter Slip (Erie Scientific; 25 μL volume) was placed over each array. Slides were then incubated with erythrocytes suspended in blocking buffer for 2 h. One percent erythrocyte suspension was used throughout, unless specified otherwise. Slides with hybridization chambers were gently mixed throughout to allow movement of the erythrocytes. Slides were submerged into PBS pH 7·0, then transferred to PBS pH 7·0 containing 0·1% Tween 20 for a 20-min washing step with constant mixing. This was followed by two additional washes in PBS pH 7·0 for 10 min. After the final wash, the slides were dried by brief centrifugation at 1000 r.p.m. Nitrocellulose slides were processed by Schleicher & Schuell recommended methods.

*Imaging and data analysis*

All slides were scanned in an Affymetrix 428 Array Scanner set to the Cy3 channel for the detection of FITC. Numerical data was extracted from the microarrays using QUANTARRAY® MICROARRAY ANALYSIS Software (GSI Lumonics, Canada). This gave a fluorescence intensity value from the centre of each spot and a background value from the area surrounding each spot. For each spot, the background fluorescence value was subtracted from the fluorescence intensity value. Each slide was scanned with at least three different photo-multiplier tube settings. A scatter plot was prepared for each slide using the signal intensity values from each scan setting set against each other. The shape of the resulting data cloud gave an indication of the scan qualities. The $R^2$ value was applied to each graph, and the scan giving the most highly optimized data was selected. A negative control population was derived from human monoclonal IgM and IgG (LDM3 and ESD1 antibodies as described in Table 1). A median of replicate spots signal/noise (S/N) ratio was then calculated and used in presented data. Use of S/N ratios meant normalization was not required between comparative slides.

*Biacore analysis*

The method used was adapted from that detailed in Quinn *et al.* (1997), with modifications: The Biacore X, Biacore CM5 chip, BIACORE X CONTROL Software and BIAEVALUATION Software were used. Data presented in this work are reference subtracted (data from a reference flow cell subtracted from data from active flow cell). Briefly, ligands were dialysed against 10 mM sodium acetate pH 5·0 and applied at a flow rate of 5 μL per minute. The concentration of ligand was 50 μg $mL^{-1}$. The surface of the chip was activated for 10 min. The coupling of ligand was performed using contact time of 10 min. The surface of the chip was deactivated for 7 min using 1 M ethanolamine-HCl pH 8·5. The analyte was prepared in HBS buffer, and interaction analysis was performed for 10 min. Response is measured in response units (RU) and is proportional to the mass on the sensor surface (one RU represents a change of 0·0001 in the angle of reflective light). NaOH (1 mM) was used for the chip regeneration.

## RESULTS

*Slide surface selection and optimization of reaction conditions*

Initial development involved a selection of slide surfaces suitable for antibody immobilization and at the same time for specific erythrocyte binding, optimal erythrocyte concentration and effect of reaction conditions such as volume and mixing (Fig. 1A–D). Additional experimental variables investigated included various blocking agents, detergents and incubation and washing times (data not shown). Figure 1A shows the specific binding of Cy3 labelled anti-human IgG to human IgG antibody and human serum while the cross-reactivity with heterologous sera and antibodies was generally low. In this type of assay, S/N ratio on the polyacrylamide slide was superior to other substrates, exhibiting around five times higher S/N than other substrates. Indeed, polyacrylamide pore size is unlikely to permit the penetration of erythrocytes used in blood typing reactions. Amino silane-, poly-L-lysine- and nitrocellulose-coated slides showed substantial but lower S/N (between 10 and 40). The lowest values of signal on heterologous probes were on poly-L-lysine slides, with nitrocellulose slides showing slightly higher cross-reactivity for certain probes.

Poly-L-lysine and polyacrylamide slides were chosen for evaluation of blood typing reactions, the polyacrylamide slides for their generally high S/N values (see below) and poly-L-lysine slides for easy

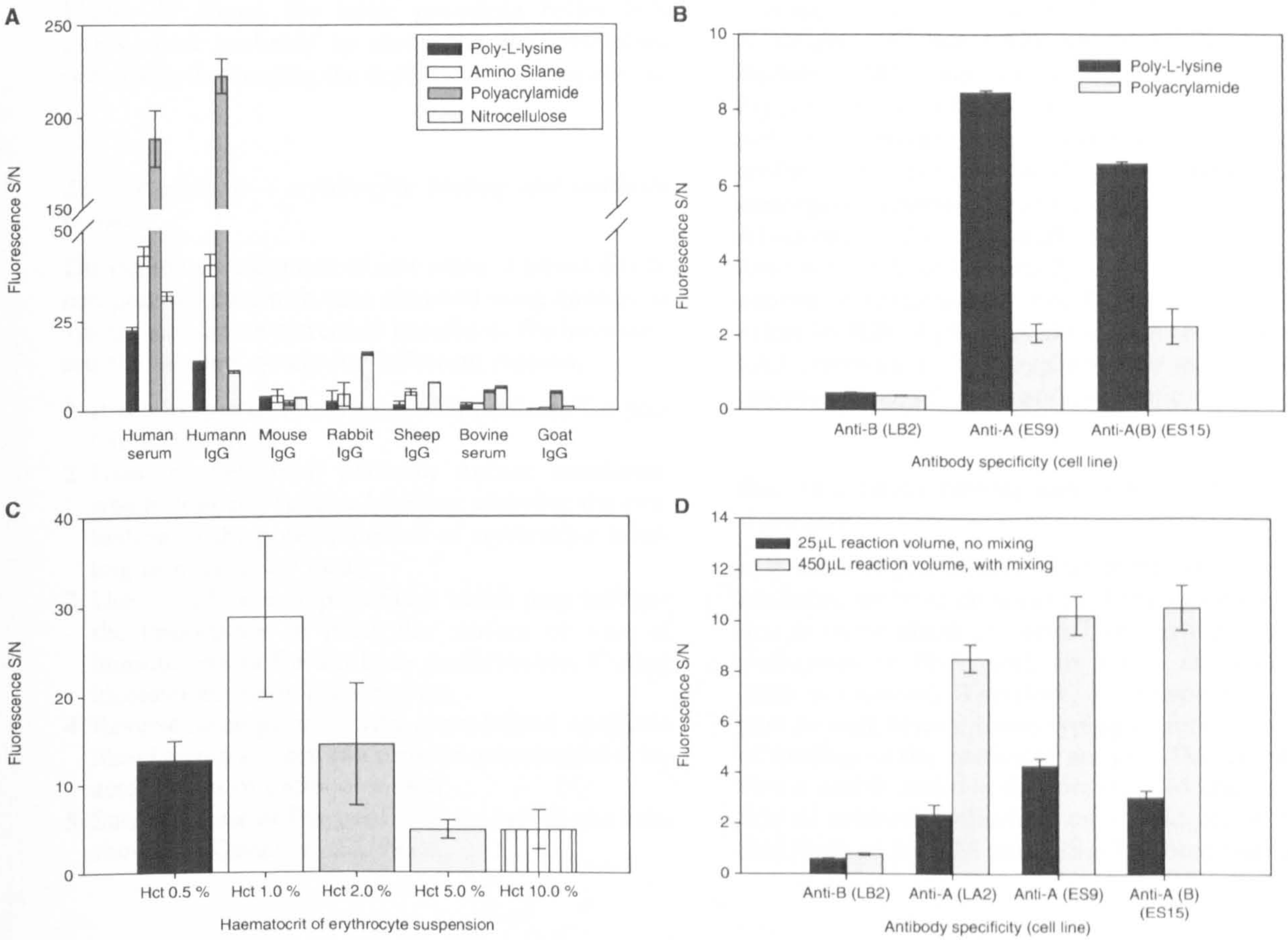


**Fig. 1.** Optimization of parameters for microarray blood grouping assay. Antibody and serum probes were printed, slides processed, scanned and data analysed as described in *MATERIALS and METHODS*. Signal/noise (S/N) ratios are the median of at least three replicates. (A) Specificity of reaction of Cy3-labelled anti-human IgG reaction with human immunoglobulin (Ig)G and mammalian sera on four different microarray surfaces. (B) Binding of fluorescein isothiocyanate (FITC)-labelled group A1 erythrocytes to blood group antibodies on poly-L-lysine- and polyacrylamide-coated slides. (C) Effect of erythrocyte concentration (haematocrit). Different concentrations of FITC-labelled group B erythrocytes were incubated with anti-B (LB2) immobilized on poly-L-lysine slides. (D) Effect of reaction volume and mixing. FITC-labelled group A1 erythrocytes were incubated with anti-A (LA2) antibodies immobilized on poly-L-lysine, using different volumes and mixing conditions.

and reproducible in-house preparation and relatively low background values. As shown in Fig. 1B, this test permitted specific detection of blood group antigen A-specific RBC with both substrates. The group A erythrocytes reacted with antibodies of anti-A and anti-A(B) specificities but not those with anti-B specificity. Of note is that the poly-L-lysine slides generated a higher S/N ratio than polyacrylamide slides, contrary to antibody–antigen interactions (Fig. 1A), presumably due to the RBC-probe ligand reaction being limited to the polyacrylamide patch surface.

A parameter that may vary depending on the number of blood group antigen sites carried by erythrocytes of the corresponding specificity is the optimal RBC concentration. We have investigated erythrocyte preparations of different haematocrits and found that for microarray-based ABO grouping the optimal RBC concentration was 1%, as shown in Fig. 1C.

Other parameters to consider for the reaction of immobilized antibody probes with an antigen on a corpuscular carrier (erythrocyte) were the reaction volume and the effect of mixing. We have compared a static incubation in a minimal volume of 25 µL under Lifter Slips™ with gentle mixing in a larger reaction volume of 450 µL using reaction chambers.

Figure 1D shows the latter providing better S/N ratios, most probably by increasing the interaction probability by keeping the erythrocytes in suspension.

*Biacore analysis – erythrocyte binding and antibody activity*

During the development of new assay, a possibility to compare the data with data obtained using analogical system may prove extremely beneficial. We have chosen the Biacore system for following reasons:

1 It is a long-established analytical platform for protein interactions;
2 Uses for detection, plasmon surface resonance, which does not require labelling allowing the evaluation of the potential effect of erythrocyte labelling in microarray assay;
3 Use of different chip surfaces which may indicate the importance of particular surface or way of immobilization for antibody performance, if using identical antibody preparations;
4 Reverse arrangement with immobilized synthetic blood group antigen can provide quantitative characterization of antibodies used;
5 Successful use of Biacore for blood typing has been shown by Quinn *et al.* (1997).

A mouse monoclonal antibody against blood group A antigen (cell line LA2) was immobilized onto a Biacore CM5 chip under optimized conditions. Erythrocytes of different groups were passed over the surface and binding reactions recorded as sensorgram profiles and as tabulated binding data. Figure 2 shows sensorgrams revealing clearly the best interaction with A1-specific cells, followed by A2-specific cells. Reactivity of $A_1$ cells is usually higher due to the higher number of antigenic sites (see *DISCUSSION*). Lower values for RBC $A_1B$ and $A_2B$ specificity corresponds to LA2 reactivity in haemagglutination assay, with no reactivity detected for B- and O-specific erythrocytes.

*Biacore antibody ranking using synthetic blood group A antigen*

In a series of preliminary experiments on the Biacore platform, we have demonstrated the specific interaction of immobilized synthetic blood group A antigen conjugated to BSA, with an anti-A antibody LA2 (data not shown). Therefore, we attempted to compare or rank several blood typing antibodies in terms of binding to the particular antigen. This could provide a useful, additional, more detailed characterization of antibody behaviour on a solid phase. Given that some of the IgM antibodies had been purified by

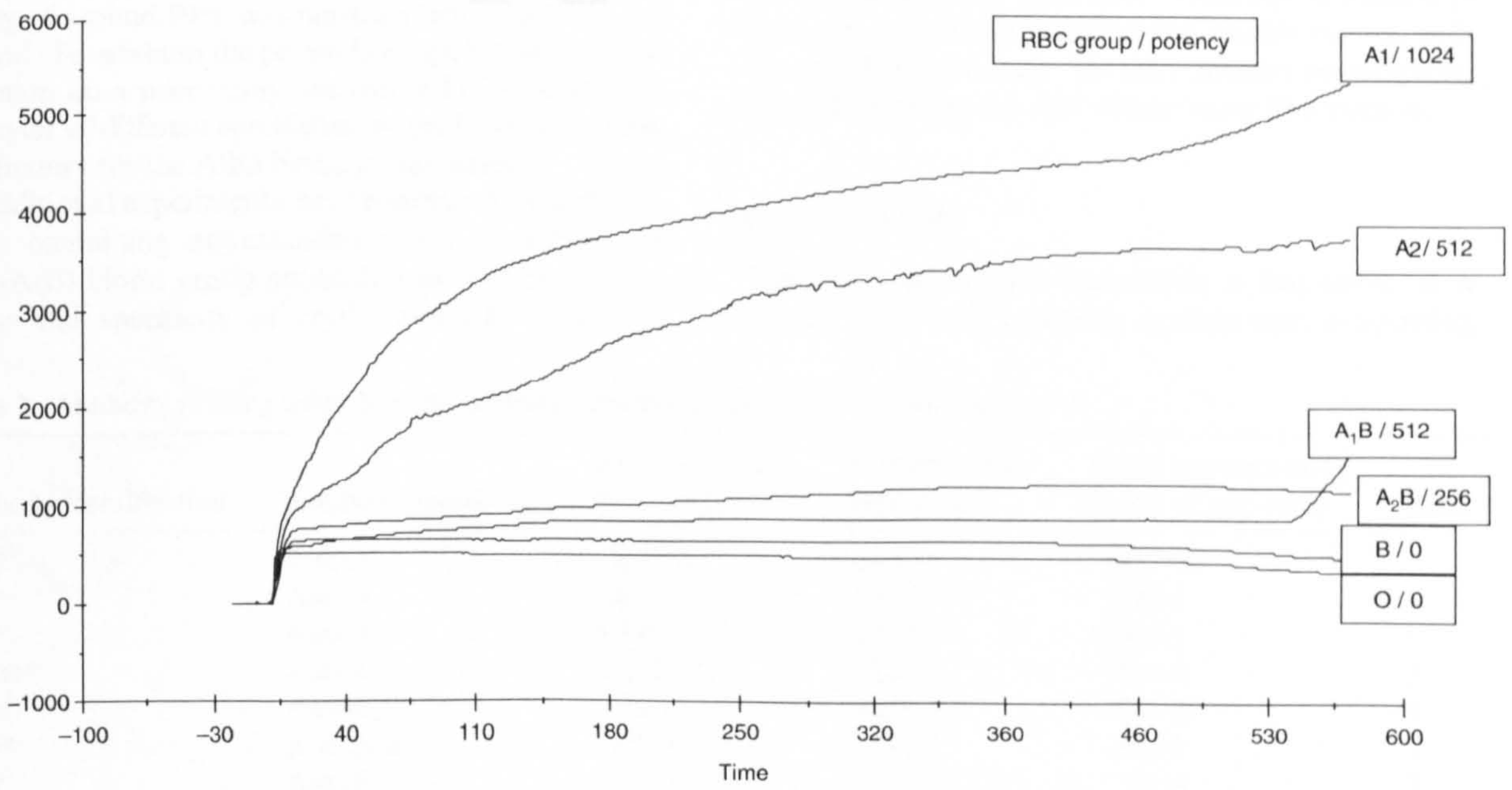


**Fig. 2.** Surface plasmon resonance sensorgram of the interactions of immobilized blood group antibody anti-A (LA2) with erythrocytes of various ABO groups (A1, $A_2$, $A_1B$, $A_2B$, B and O) as determined by Biacore analysis. Potency of the anti-A (LA2) against each erythrocyte as measured by haemagglutination is shown next to the corresponding curve.

two different methods, it could also serve as a comparison of functionality when the antibodies have been processed differently. Synthetic blood group A antigen
14 conjugated to BSA (Dextra Laboratories) was coupled to a CM5 chip. Selected blood group antibodies were sequentially injected over the sensor chip surface. Table 2 lists the response and mean response from two runs in RU. All anti-A antibodies (ES9, LA2 and DAM1) showed the strongest reactivity, almost two times (three times in the case of ES9) of that for anti A(B) antibody ES15. Anti-B antibody, LB2 showed no reactivity. It can also be seen from Table 2 that antibodies purified with two different methods gave similar results, although affinity-purified antibodies exhibited slightly increased values. Biacore chips can be used repeatedly, and the data presented show the means of two runs which were highly reproducible, showing a difference of no more than 7%. Quantitative measurements provided by Biacore represent useful characterization of reagents to be immobilized on a solid phase.

*Microarray-based blood typing*

A panel of antibodies specific for the ABO blood group system were arrayed onto poly-L-lysine slides at a range of concentrations. The optimal detection method used in the development stage of the assay was direct labelling of erythrocytes with FITC. FACS analysis was used to determine the degree of labelling. High labelling efficiency of around 99% was normally achieved (data not shown). To establish the principle of specific erythrocyte detection on a microarray, we used FITC-labelled ery-
15 throcytes of different specificities to probe the array discriminatory for the ABO blood group system.

Additional experiments were conducted on a microarray containing an extended panel of anti-A, -B and -A(B) blood group antibodies to further increase range and specificity of erythrocyte-type detection. Figure 3 shows data from a typical experimental run demonstrating the specific detection of the four main ABO group erythrocytes on a poly-L-lysine-based array format. Antibodies were reacted with directly fluorescently labelled RBC preparations of each of the four main blood group specificities. Group O cells have neither A or B antigen present and they effectively serve as a negative control. Anti-A, -B and -A(B) antibodies exhibit specific reactivities with S/N between 38 and 96. Somewhat surprisingly, anti-A(B) antibody ES15 outperforms the single specificity anti-A (LA2) and anti-B (LB2) antibodies in reactivity against A- and AB-specific RBC, although reactivity with B-specific erythrocytes was minimal. The cell line ES15 is known to react only very weakly with group B RBC (Moore *et al.*, 1984). Although giving similar results to the other probe antibodies, the concentration of anti-A (LA2) is far higher (about four times more concentrated). Further technological improvements using an extended range of anti-blood group antibodies have been investigated in a parallel study.

The labelling of each individual erythrocyte preparation is, however, impractical for high-throughput screening. We have therefore investigated various secondary FITC-labelled reagents [anti-Rh29 antibody and wheat germ agglutinin (WGA) lectin]. Figure 4 shows the detection of bound A1-specific erythrocytes with FITC-labelled WGA and anti-Rh29. The achieved S/N ratio (3–5) was lower, in some cases considerably lower, than that of directly labelled erythrocytes, albeit still sufficient for specific typing, with the specificity retained. We are currently investigating ways to improve the S/N ratios using this method.

## DISCUSSION

The microscope slide has made a full circle as a matrix for blood grouping applications, supporting

**Table 2.** Antibody ranking using Biacore chip-immobilized synthetic blood group antigen A

| Antibody identification | Antibody specificity | Response units (first run) | Response units (repeat run) | Mean response units (mean of two runs) | Rank |
|---|---|---|---|---|---|
| LA2† | Anti-A | 9267·8 | 8431·1 | 8849·45 | 2 |
| LA2* | Anti-A | 8114·3 | 7573·9 | 7844·1 | 4 |
| ES9* | Anti-A | 12393·5 | 12503·1 | 12448·3 | 1 |
| DAM1† | Anti-A | 8112·7 | 7816·3 | 7964·5 | 3 |
| ES15† | Anti-A(B) | 4605·1 | 4158·6 | 4381·85 | 5 |
| ES15* | Anti-A(B) | 4369·0 | 3795·6 | 4082·3 | 6 |
| LB2† | Anti-B | 83·1 | 39·7 | 61·4 | 7 |
| LB2* | Anti-B | −103·5 | −177·8 | −140·65 | 8 |

*Gel filtration purified.
†Affinity chromatography purified.

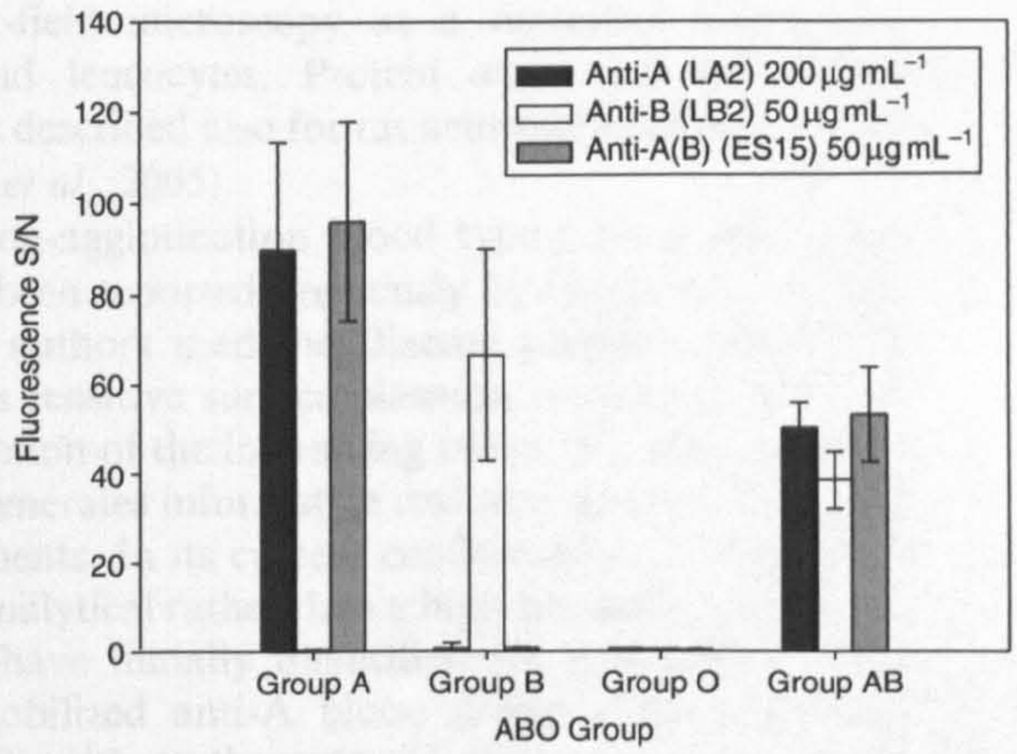


**Fig. 3.** Microarray blood grouping with directly labelled erythrocytes. Fluorescein isothiocyanate (FITC)-labelled erythrocytes carrying antigens of different specificities (A, B, O and AB) were incubated with blood group specific antibodies printed onto poly-L-lysine slides. Signal/noise (S/N) ratios as Fig. 1.

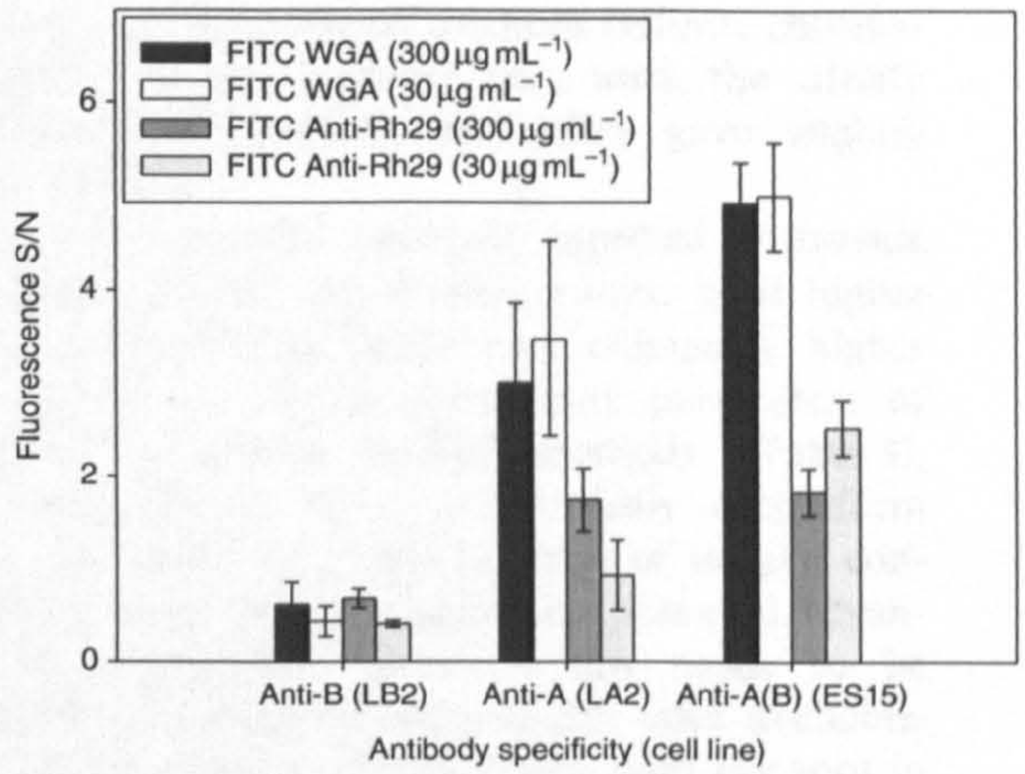


**Fig. 4.** Microarray blood grouping determined by indirect labelling of bound erythrocytes. Group $A_1$ erythrocytes were incubated with blood group antibodies immobilized on poly-L-lysine-coated slides and then with fluorescein isothiocyanate (FITC)-labelled wheat germ agglutinin (WGA) or FITC-labelled anti-Rh29 monoclonal antibody. Signal/noise (S/N) ratios as Fig. 1.

a single antibody–single blood sample assay a few decades ago and a multiple antibodies–one blood sample (one chip–one donation format) assay today. Miniaturization reduces consumption of the probes and samples and decreases reaction times. These assay parameters are primarily important in mass screening application such as blood typing in a blood bank setting. In this study, we have investigated if the benefits of miniaturization as seen in a whole genome analysis could be applied to this protein-based diagnostic technique.

Antibodies immobilized on a solid phase have been used in blood typing either in a microplate format or in gel cards, although in the latter case the antibodies are suspended rather than attached. In both cases, the interaction with red cells leads to agglutination, automated readout of which may sometime be problematic (Scott, 1991). An individual well of a microplate or a microtube within the gel card both contain single specificity antibody. Planar (or open plan) microarrays prepared by contact or ink-jet printing are more suitable for multiplexing, as the number of probes is highly flexible. We have investigated parameters such as the antibody concentration, various spotting surfaces and blockers, sample dilution, etc. Although polyacrylamide-coated (Hydrogel) slides were superior in a classical antibody–antigen type reaction, mainly due to high capacity, this advantage was lost in a blood typing reaction. Hydrogel pores are impenetrable to very large molecules such as IgM and also to erythrocytes and other cells. It is likely that with polyacrylamide, interactions are limited to the polyacrylamide patch surface, perhaps slightly bigger than normal two-dimensional spot due to its shape but still not sufficient to compensate for a loss of internal patch capacity. Poly-L-lysine slides provided a satisfactory and cost efficient matrix for ABO typing. We have opted for rather large printing pins, 700 μm in diameter, to ensure sufficient numbers of erythrocytes can bind to immobilized antibodies. Sensitivity does not seem to be a critical parameter in basic ABO typing as a 700-μm spot can theoretically accommodate $4–5 \times 10^3$ erythrocytes (7·8–8·3 μm in diameter) with $2·5 \times 10^5–1 \times 10^6$ antigenic sites per erythrocyte (Daniels, 2002). However, it could be more important for antigens like $A_x$ with around 1400 antigenic sites or weak D with only 200 sites per erythrocyte. We have investigated additional surfaces and an extended range of blood group antigens in a parallel study. The reaction between an immobilized antibody and antigens carried on the cell surface is different from the reaction with a soluble antigen. The role of antibody affinity is perhaps more important for retention of specifically attached red cell through the antigens on its surface, especially during the washing procedure. Protein–cell interaction studies using microarrays are still uncommon, and we believe this is the first comprehensive study showing the feasibility of blood group typing in this format. An analogical study using antibody arrays for leucocyte immunophenotyping has been described by Belov *et al.* (2001), although in a slightly different set up, using nitrocellulose slides and a non-conventional

dark-field microscopy as a detection method for bound leucocytes. Protein array cell interactions were described also for rat neuronal stem cell analysis (Ko *et al.*, 2005).

Non-agglutination blood typing on a solid phase has been reported previously by Quinn *et al.* (1997). The authors used the Biacore platform which provides sensitive surface plasmon resonance, label-free detection of the interacting molecules. Biacore analysis generates informative real time quantitative measurements. In its current configuration, however, it is an analytical rather than a high-throughput platform. We have initially quantified the interactions of an immobilized anti-A blood group antigen antibody (LA2) with erythrocytes of various specificities and obtained a pattern of reactivity matching that of soluble haemagglutination with the same antibody. Indeed, several antibodies (e.g. LA2, ES9 and ES15) have been used in three parallel assays: haemagglutination, Biacore and microarray. We have also used the Biacore chip in a reverse assay with immobilized BSA-conjugated synthetic blood group A antigen for ranking various ABO typing antibodies. Interestingly, we observed differences in reactivity patterns of certain probe antibodies in different assay formats. While anti-A antibodies such as LA2 or ES9 are used at same potency as anti-A(B) ES15 in haemagglutination assays, ES15 reacted less well with a synthetic blood group A antigen immobilized on a Biacore chip. However, ES15 usually outperformed ES9 and LA2, when immobilized on a microarray. This type of comparative data will be useful for defining choice of surface, immobilization technique and antibody probes for microarray-based blood group serology. Where IgM antibody preparations purified with two different methods (affinity chromatography and gel filtration) were used, the affinity chromatography-purified antibodies gave slightly better signal.

The new assays are generally expected to provide an advantage over the current assays, be it higher sensitivity/specificity, better cost efficiency, higher throughput, etc. When considering parameters of different serological testing methods (Table 3), microarray blood typing could easily outperform other currently used assays in terms of reagent consumption and there is a significant potential advantage in throughput, although this needs to be confirmed on an automated system once available. Even the 20 nL of antibody probe used per spot in this study can be greatly reduced with smaller diameter printing pins. Compared with the other methods, the antibodies used in microarrays were purified, and it remains to be seen how important the purification step really is for microarray blood grouping and by how much it would affect the savings made on reagent consumption. Multiplexing power, however, is what sets the microarray format apart from the other systems.

We describe in this paper microarray-based ABO blood grouping, as the first step in the development of a comprehensive microarray platform for blood donation screening. This work is being extended to other blood group antigens and antibody screening work, as well as assays that would combine at least some aspects of blood group serology with microbiology testing. One chip–one donation concept of screening would greatly simplify the testing algorithms. This concept could be extended to a more logistically demanding one chip–multiple donations

**Table 3.** Parameters of different blood grouping techniques

| Method | Ab* probe volume (μL) per data point | Sample volume (μL) per number of probes | Readout method | Throughput: number of samples (data points) | |
|---|---|---|---|---|---|
| | | | | per run | in 6 h |
| Manual/tube | 40 per test | 40 per 1 | Haemagglutination | 50 (350)† | 300 (2100)† |
| Microplate | 25 per well | 25 per 1 | Haemagglutination | 240 (2880) | 1440 (17280) |
| Column agglutination | 25 per tube | 50 per 1 | Haemagglutination | 70 (420) | 420 (2520) |
| Biacore chip | 70 per chip | 70 per 1 | Surface plasmon resonance | 1 (1) | 24 (24) |
| Planar protein microarray | 0·02 per spot‡ | 25–450 per $n \times 10$ to $n \times 100$ | Fluorescence | $n \times 10$ ($n \times 1000$)§ | $n \times 100$ ($n \times 10000$)§ |

*All antibodies are used at similar titre (between 1 : 256 and 1 : 512]. Purified antibodies were used for Biacore and microarray analysis.
†Estimate.
‡For 700-μm solid pin. The volume can be reduced significantly using smaller pins or ink-jet printing.
¶Estimate.

algorithm, technically achievable in a microarray format. More immediate extension of blood typing arrays is, however, likely to be a combination with antibody screen probes and/or with probes relevant for blood-borne pathogens screening, both approaches being actively pursued by this group.

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Microarrays and blood diagnostics

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Content



**Summary**

Microarray technology has a potential to alter many diagnostic applications, especially high throughput multiparameter assays. In this paper we discuss potential impact of microarrays on blood donation screening algorithms by providing a single testing platform for microbiology and blood group markers. An initial stage of such development is demonstrated on examples of successful blood grouping and antibody screen for some rare alloantibodies.

## 1. Current blood testing methods

Blood tests can reveal a variety of changes taking place during disease processes, as well as genotype and phenotype characteristics necessary for further medical interventions. There are a huge number of tests and instruments in use, reflecting the needs of specialised hospital laboratories as well as individual point of care devices. Microarrays could play an important role predominantly in high throughput applications. In this paper we focus on the potential role of microarrays in future blood donation screening techniques.

### 1.1. Unique features of blood donation screening

Blood transfusion occupies rather an unusual place among medical procedures in the level of expected safety of both the procedures and the outcome. Risk assessments for very few other medical procedures would be comparable to figures for residual risk of transfusion-transmitted viruses, currently in the range of one in several million of donations (1). Combination of donor selection methods, testing procedures and, in some cases inactivation techniques guarantees an extremely safe blood supply (2).

Blood donation screening is a unique process for several reasons:

a) the vast majority of donations will produce a negative result for blood borne pathogen because blood donors represent a self-selected healthy population;

b) each sample positive for blood borne pathogens must, however, be detected because blood, blood products and components are destined for recipients. The sensitivity of the screening test needs to be extremely high, even at the expense of slightly lower specificity. Repeat testing is a routine component of testing algorithms;

# Appendix 4

c) for the same reasons, clinically relevant blood groups and the presence of blood group alloantibodies have to be reliably determined;

d) screening procedures have to be able to satisfy throughput needs between several hundreds and a few thousand donations per day (for an average size blood centre);

e) turnaround time has to be short due to the shelf life of some of the components (e.g. five day shelf life for platelets) and flexibility of stock management.

### 1.2. Testing targets

Each donation has to undergo testing for a set of markers for selected blood borne pathogens and to determine blood group compatibility. Table 1 lists the testing targets and parameters of currently used testing procedures.

#### 1.2.1. Testing for blood borne pathogens

Highly sensitive immunoassays form the basis for the majority of microbiology assays. They usually represent various modifications of sandwich ELISAs. Ideally, the screening would be based on direct antigen detection, confirming the presence of an infectious agent. In many cases, however, the antigen concentration or titre of the infectious agent in circulating blood is too low to be detected in this way. One exception is hepatitis B surface antigen (HBsAg) produced at quantities sufficient for robust antigen detection. The best HBsAg assays can detect around one pg /ml which is approximately $8.35 \times 10^6$ molecules for 24 kDa protein.

In most cases we are measuring antibodies developed as a response to infection. The appearance of measurable antibodies may take a significant amount of time creating a potentially long window period.

For immunoassays, the antibodies (polyclonal or monoclonal) or antigens (recombinant proteins; peptides) are attached to a solid phase, most frequently represented by the surface of a bead (microsphere) or a microplate well. One or two step sandwich detection is usually used, with conjugated enzymes converting substrate into colour or (chemi) luminiscent signal.

Due to the sensitivity issue with many antigen-detecting immunoassays, much hope was placed in the application of nucleic acid amplification technologies (NAT), most frequently represented by the polymerase chain reaction (PCR). This technology indeed has an exquisite sensitivity (Table 1) and detects the component of the infectious agent rather than antibodies. The window period for viruses for which PCR was introduced as a routine screening method, has been shortened significantly and residual risk of transfusion transmitted events are extremely low. It would seem to be an ideal screening procedure. However, the cost efficiency of this type of testing is being increasingly questioned, especially as the number of NAT only positive donations is lower than originally expected (see 1.3.).

#### 1.2.2. Blood typing and detection of alloantibodies

The traditional method of monitoring blood group serology reactions is haemagglutination. It is an inexpensive technique with easily identifiable end point detection. There are two basic types of assays both included in routine testing procedures. In "forward blood typing" the red blood cell (RBC) antigens of clinically relevant blood group systems are determined using specific polyclonal (minority) or monoclonal antibodies (MAb). IgM MAb are preferred to IgGs as they cause haemagglutination in one step thanks to their structure - providing 10 antigen binding sites per molecule compared to two per IgG molecule. Microplate assay format is most frequently used for donation blood typing (Table 1). "Reverse typing" is designed to detect antibodies against blood group antigens. The reason for development of alloantibodies (or isoantibodies) is usually Ab pregnancy, blood transfusion or some immunisations. Clinically relevant antibodies need to be detected in donors and, especially in recipients as they could cause life-threatening reactions. The microplate haemagglutination is the most frequently used detection method for donation testing, while lower throughput patient testing is most often done using gel cards.

### 1.3. Advantages and disadvantages of current testing methods

As mentioned earlier, current preventative screening and testing procedures used in developed countries provide an extremely safe blood supply. Introduction of a conceptually different method in the form of PCR into a routine testing algorithm for certain blood borne pathogens ended an era completely dominated by immunoassays. It makes it easier for blood centres to accommodate improved and new technologies as they emerge.

PCR would be a very attractive general testing platform due to its sensitivity and precision. PCR and other target amplification techniques have been used not only to detect pathogens but also for determination of RBC and platelet antigens (3-6). Unfortunately, there is a high cost attached to PCR screening, both direct (contamination prevention measures, reagents, instrumentation, specialist staff) and indirect (significant license fees etc.). The only viable way to achieve satisfactory cost efficiency would be extensive multiplexing. While some multiplex assays

were successfully developed, the number of targets is generally low. This is mostly due to an interference of primers and probes, difficult to predict even with the help of oligonucleotide design software. Future improvements may, however increase the multiplexing potential of target amplification methods. At the same time there is a continuous improvement in the performance of immunoassays and some antigen or combi (antigen and antibody) assays are getting closer to NAT assays in respect of closing the window period (7). It does not seem probable at present that one type of assays (nucleic acid based or immunoassays) would be able to replace the other type entirely. It remains to be seen how successfully they can be co-developed in the future.

It is fair to say we have an extremely safe but also a complex testing algorithm in place. Microarray technology could address this complexity by providing a single donation testing instrumental platform for a complete set of required markers (8; see below).

**2. Microarrays as the potential next generation testing platform**

First experiments to further miniaturise the existing immunoassays date back to late eighties- early nineties (9). In parallel, several groups were developing techniques for simultaneous monitoring of large groups of genes. These techniques required high probe density facilitated by the use of new, non- porous materials such as glass, silicon or plastic, allowing for the reduction in the quantities of deposited probes and reaction volumes. Robotic spotting techniques (10,11) and in-situ oligonucleotide synthesis (12) speeded up gene expression studies with information content provided by large sequencing projects. Significant efforts were dedicated to developing new surface chemistries as well as alternative microarray formats including encoded beads and exploiting microfluidics and additional features such as electronic probe and sample addressing (13-15). While driven mainly by genomic research, the potential of microarrays for diagnostics has been quickly identified. In fact, diagnostics may well become the main microarray application in the future.

It soon became clear that some of the techniques used for preparation of DNA arrays could be adapted to protein microarrays (16,17) despite the more complex character of protein interactions compared to rules for complementary nucleic acid strands re-annealing. Alternative systems describing the use of polyacrylamide patches and subarrays within microplate wells were also reported (18,19).

For mass screening applications such as blood donation testing the cost efficiency is one of the main parameters to consider, together with high sensitivity and specificity, robustness and a high level of automation, limiting or eliminating the operator-introduced errors. This will inevitably affect the complexity of future microarray based testing platforms. An open plan blood typing microarray, with no physical barriers between the probes, has the potential to develop into a highly cost-effective high throughput system for blood screening if it can sustain the specificity of reactions and achieve the required sensitivity.

Most protein arrays described to date investigate the interaction of free protein molecules with the defined partner (probe) immobilised on the solid surface. Probes are represented predominantly by antibodies (18-21), is some cases by antigens (22,23). Blood group serology adds another dimension to these interactions as the interaction involves blood group antigens on the surface of red blood cells (RBC). Antibody - cell interaction on planar microarrays have been described for applications such as leucocyte typing (24) but still are rather rare. Quinn *et al.* (25) described solid phase blood grouping using the Biacore platform but this is an analytical rather than high throughput application platform.

**2.1. Potential benefits of microarray testing**

In the currently used testing algorithms, several aliquots of donated blood are taken to be used on between three and six instrumental platforms to test for a complete set of required markers (see 1.2.1. and 1.2.2.). Time required for testing on different instruments varies and sets of results become available at different times, the slowest holding up the release of blood and blood components which cannot be issued without final data reconciliation. A proportion of samples will undergo repeat testing as the sensitive tests necessary for initial screening produce some false positive samples in addition to true positives. Table 2 provides a comparison of current algorithms with potential changes, which might be possible, for future microarray based testing platform. The most significant change would be transition from testing many samples in parallel for one or few markers on multiple instrumental platforms, to simultaneous testing of each sample for the required set of markers on 1 -2 chips. The IT side of the operations would be simplified by data provided simultaneously. One platform would not require multiple sets of reagents for various instruments or staff dedicated to individual platforms, as is the case at present. Inclusion of multiple probes per target is easier in microarrays as the incremental cost of additional probes is low. This could perhaps eliminate the need for repeat testing, as the reactivity pattern on multiple probes should provide a clear answer on sample reactivity.

## Appendix 4

### 2.2. Options to increase microarray sensitivity

Sensitivity is probably the major problem for wider use of microarrays for some diagnostic purposes. This problem is often being addressed by combining target amplification (e.g. PCR) using degenerate primers or non-specific amplification, with subsequent microarray analysis (26-28). Such combination would add another extra step to existing blood donation testing algorithms, increasing the complexity of testing as well as costs. Fortunately, there are multiple alternative approaches currently being developed to increase the detection limits.

Ideally, the screening assays would approach the PCR sensitivity at lower cost, effectively through the higher multiplexing power, and perhaps the true multianalyte character of the procedures. Current PCR techniques allow detection of less than 10 genome equivalents per reaction, amplifying the target around billion times. The best ELISA based assays can detect $10^6$ - $10^7$ molecules per ml. How can this gap of around six orders of magnitude be closed in the absence of target amplification methods for proteins? Certain advantage is offered by the presence of hundreds to thousands of copies of the viral antigens compared to 1-2 copies of genomic RNA or DNA per virion. In addition, there are some alternative methods of signal enhancement as listed in Table 3. Signal amplification methods seem to provide the biggest gain in sensitivity in comparison to usual methods using few fluorophore molecules conjugated to primary or secondary reagents in sandwich assays. In order to apply any amplification method simultaneously to all sites on a planar microarray, the amplified signal needs to be localised, not diffuse. One primer variant of rolling circle amplification (RCA; 29) produces a long product physically attached to the site and can provide signal amplification exceeding $8x\ 10^3$ (30). Another amplification method is tyramide signal amplification (TSA) based on the catalytic activity of horseradish peroxidase (HRP) generating high-density labelling of a target claiming sub pg/ml sensitivity. Elimination of the need for physical separation of individual sites necessary for target amplification methods makes these techniques less expensive. Other advantages include use of RCA and TSA for both, DNA and protein arrays (31, 32) and isothermal reaction.

A smaller, but significant increase in sensitivity is provided by new detection techniques. Resonance light scattering (RLS) uses a white-light source scanner to detect monochromatic, scattered light signals of metal (gold, silver etc.) particles. The material, size and shape of the particles determine the signal. When RLS was directly compared to fluorescent detection of bacterial pathogens, the authors observed even 50-times more intense signal (33).

Planar waveguide technology developed by Zeptosens utilises a special coating of $T_2O_5$ or $TiO_2$ to induce evanescence field for efficient fluorescence detection (34), at least 10 times more sensitive than the conventional fluorescence if using the company's chips and a reader.

New materials such as quantum dots (or semiconductor nanoparticles) provide brighter fluorescence, narrower emission spectra and higher resistance to photobleaching compared to the conventional small molecule dyes. Signal can be increased, however also using small molecule dyes enclosing thousands of dye molecules within a derivatised nanoparticle and enhancing the signal many times (35).

Instead of using a linear probe, usually labelled with a single fluorophore, dendrimers or multiply branched molecules provide the opportunity to introduce many dye molecules and to increase generated signal. When used for DNA microarray human herpes virus diagnosis, the signal has been enhanced at least 30 times (36).

Some of the described approaches can be further combined leading to very sensitive assays, potentially approaching the sensitivity of nucleic acid amplification techniques. Combined with easier multiplexing such assays could be well suited for high throughput blood screening of the future.

## 3. Development of microarray blood testing format

As outlined in Table 2, a microarray-based blood screening platform could provide advantages over existing testing algorithms. An ultimate testing platform would combine pathogen testing with blood group serology. As the first step in this development we have investigated the applicability of blood grouping in a microarray format.

### 3.1. Blood group serology

The vast majority of blood serological methods are based on the ability of erythrocytes to agglutinate. Reactions in the liquid phase using tubes was replaced by slide and tile reactions and later by microplate format. Solid phase assays (37,38) were more suitable for pre-dispensed dried reagents and automation, including operator-independent readout. An alternative solid phase assay is the gel card system containing microtubes with antibodies suspended within a gel or glass microbeads (39, 40).

Agglutination techniques on solid phase have been exploited with lesser success in the reverse format to test for clinically relevant alloantibodies against blood group antigens. It proved more difficult to reproducibly

immobilise and store erythrocytes or erythrocyte ghosts than immobilised antibodies (41, 42), and despite various systems being developed gel cards are still the most widely used format.

The experiments described below aimed at developing an array platform for the determination of the antigens of main blood group systems using a set of well-defined proprietary antibodies routinely used in current agglutination-based assays.

### 3.2.1. Blood grouping

Microarray blood grouping could replace an agglutination reaction with fluorescent or other quantifiable readout signals. Affinity of immobilised antibodies needs to be sufficiently high to keep specifically bound RBC attached during the incubation and washing steps. In addition, the reaction conditions must guarantee preserving the integrity of the RBC necessary for binding of the labelled RBC or fluorophore-conjugated secondary reagent. We have investigated multiple parameters of these interactions on a variety of slide surfaces under various printing regimes, reaction conditions etc. An extensive set of well functionally characterised proprietary antibodies, used in a variety of current blood grouping assays, has been exploited in these studies to be published elsewhere (Robb et al., submitted; Campbell et al., In preparation). We have shown that a reproducible ABO grouping can be achieved and successful typing can be extended to Rhesus (D, C, c, E, e), Kell and other clinically relevant blood group systems. Table 4 lists the most clinically relevant of the 25 blood group systems and shows the basis of their characteristics. Even a selection of most important of blood group systems reveals the structural variability of blood group antigens as well as large differences in their abundance. When adding the functional heterogeneity and various modes of attachment to RBC membrane affecting the access to antigens, the character of various interactions involving antigens of blood group systems could be studied by microarrays, apart from purely diagnostic application.

Figure 1 depicts a typical blood grouping experiment conducted on gold slides. Monoclonal antibodies specific for A, B, A(B), Rhesus D and K blood group antigens were immobilised on gold slides and their reactivity investigated separately with RBC of different phenotypes in respect of the above mentioned blood group antigens. In Fig 1A RBC carrying blood group A antigen bound to both anti-A and anti A(B) antibodies and RBC carrying both, blood group A and B antigens bound in addition to anti-B antibody, as expected. RBC carrying blood group antigen B bound to anti-B antibody. They could be expected to react as well with anti A(B) antibody, but reaction with this unique, single available antibody of this specificity is known to be weak even in normal heamagglutination assays (46). RBC of blood group O specificity do not, of course, react with any of the antibodies as they do not carry A or B antigens and serve at the same time as a negative control. Various levels of reactivity can be seen in Fig 1B with RBC carrying two (DD) one (Dd) or no (dd) copy of the RhD antigen. Again RBC of dd phenotype serve at the same time as a negative control in this assay. Fig. 1C shows a dose dependent signal produced by binding homozygous (KK) and heterozygous (Kk) cells to the immobilised K specific antibody. All other four RBC preparations are K negative (kk) and show no or minimal reactivity. As pointed out earlier, we were assaying two different types of blood group antigens in this experiment - carbohydrate A and B blood group antigens and protein Rhesus D and K antigens (Table 4).

### 3.2.2. Antibody screen

Another part of blood group serology is the determination of alloantibodies against blood group antigens, which may cause significant, sometime life-threatening situations if undetected in donors and especially recipients. These assays are more difficult to adapt to a solid phase, as they require immobilisation of red cells, although alternative systems can be developed.

During the initial stage of microarray antibody screen development we have immobilised RBC of various specificities on planar microarray slides modified with different surface treatments. Fig. 2A shows that signals produced by specific binding of monoclonal anti-A and anti-B antibodies to gold slides-immobilised RBC carrying corresponding antigens are several times higher than those caused by non-specific binding and/or cross-reactivity and can be clearly distinguished. Fig. 2 shows an initial series experiment under conditions not fully optimised yet. The reaction parameters are being continuously improved to reduce non-specific signal. However the background will always be higher with immobilised whole cells than isolated antigens. It can be documented by using the synthetic blood group B antigen (last column), producing lower signal to noise ratio for specific (anti-B) reaction but no cross reactivity or non-specific signal. Much lower S/N values were obtained for anti-D monoclonal antibody binding to immobilised RhD+ RBC (Fig 2B). Again, the signal on RhD- cells is detectable but significantly lower than on RhD+ cells. As in Fig1, in this case we were also measuring the reactivity of two compositionally and structurally different blood group antigens. It is known that carbohydrate A and B antigens protrude from the surface of RBC

and easily accessible, while access to protein Rhesus D antigen with epitopes close to the membrane surface is more difficult. As in other types of blood group assay this could explain the differences in the obtained S/N values. Another important factor is, of course the number of antigenic sites per RBC (Table 4).

In addition to higher background on immobilised whole cells, the stability of such reagents is a cause for concern. As an alternative, recombinant blood group antigens could be used and this approach is a subject of intense development. However, some of the antigens are multi-pass transmembrane proteins making it difficult to adopt proper configuration preserving the conformational epitopes. Linear epitopes, on the other hand can often be mimicked by synthetic peptides. Immobilisation of these probes instead of red cells provides an alternative way for antibody screening in the future.

**4. Future developments**

A complete set of currently used reagent panels for blood grouping and antibody screening needs to be evaluated on real samples to confirm applicability of a planar microarray to this type of assay. With colleagues from the Centre for Genomic Technology and Informatics (GTI), University of Edinburgh we are working on adapting mandatory blood borne pathogen tests to microarray platform. While blood group serology usually does not suffer from sensitivity problem, it can be an issue with some antigens present in low numbers on the surface of RBCs. As mentioned earlier, it is a much more pronounced problem for pathogen detection and we envisage exploiting signal enhancing methods to overcome insufficient sensitivity in some cases. Signal amplification methods could be used for both weak blood grouping and pathogen detection. In addition, these techniques could be applied to both DNA and protein arrays, should both types be necessary for complete blood donation screening. Although we have focused on protein microarrays in this paper, there is an extensive development work going on for blood group genotyping. It is quite possible that no single type of assay, DNA based or protein based will be able to completely replace the other type. This is due to some genotype - phenotype discrepancies as well as existence of certain protein only agents such as prions. Microarray technology has, however potential to accommodate all required assay formats on one testing platform.

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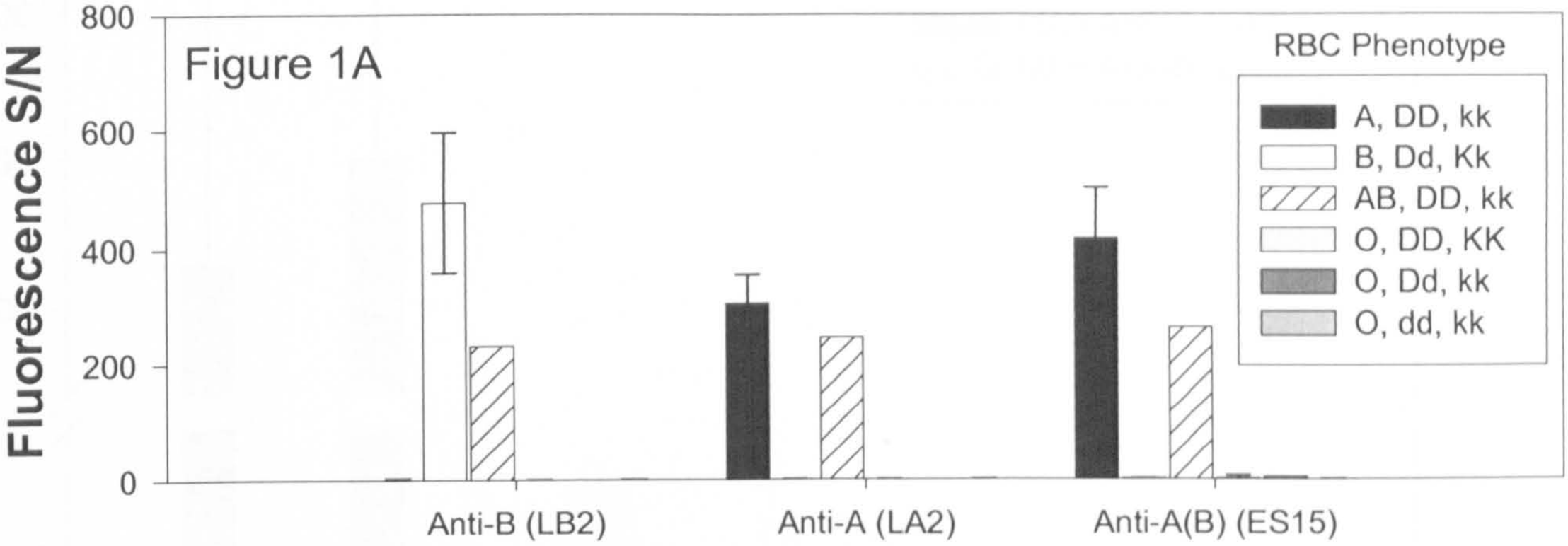
# Appendix 4

Figure Legends.

Figure 1. Microarray red cell grouping. Monoclonal antibodies specific for blood group antigens A and B (1A), Rhesus D (1B) or K antigen (1C) were printed using MicroGrid II Arrayer (BioRobotics) on gold slides (Eire Scientific), blocked and incubated for 1 hr at room temperature with 1 % suspension of fluorescently labeled erythrocytes of six different phenotypes: ABO: cells carrying A, B, both (AB) or none (O) antigen. DD cells carry two copies of D antigen, Dd one and dd none. Similarly, KK cells carry two copies of K antigen, Kk one and kk none. Slides were washed twice with PBS briefly spun and scanned on Packard Bioscience ScanArray 5000 using QuantArray® Microarray Analysis Software (GSI Lumonics). Reactivity is expressed as signal to noise ratio (S/N), with signal from PBS spots providing noise values. Values represent median of at least 3 replicates.

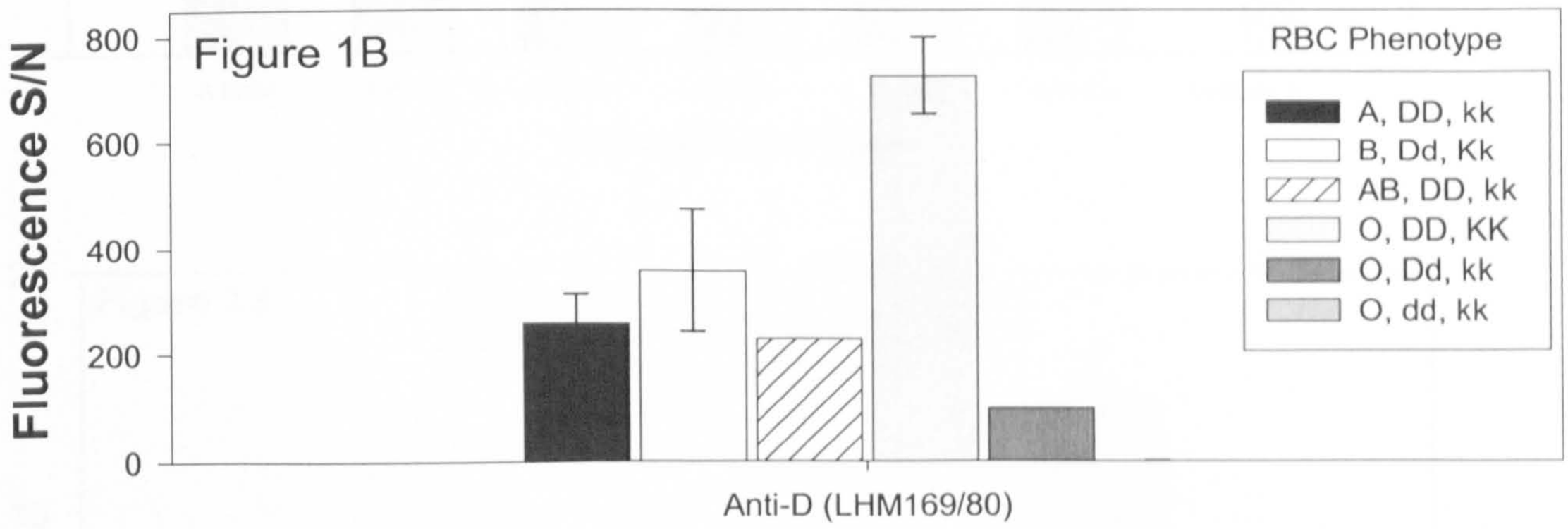
Figure 2. Microarray blood group antibody detection. Group A, B or O red blood cells, positive (RhD+) or negative (RhD-) for Rhesus D antigen were spotted on gold slides (Erie Scientific Company), alongside the synthetic blood group B antigen (Dextra Laboratories) using manual spotter (V&P Scientific) with solid pins. Slides were incubated for 1 hr at room temperature with monoclonal antibodies for blood group antigens A and B (Fig 2A) or Rhesus D antigen (Fig 2B). After repeated washing the slides were incubated with FITC anti-mouse IgM (Sigma) at 33.3 μg/ml (Fig 2A) or Cy3 conjugated anti human IgG (Sigma) at same concentration (Fig 2B), repeatedly washed , briefly spun and scanned using Axon 4100A scanner and Axon GenePix Pro 4.1. S/N values as in Fig 1.

Figure 1A



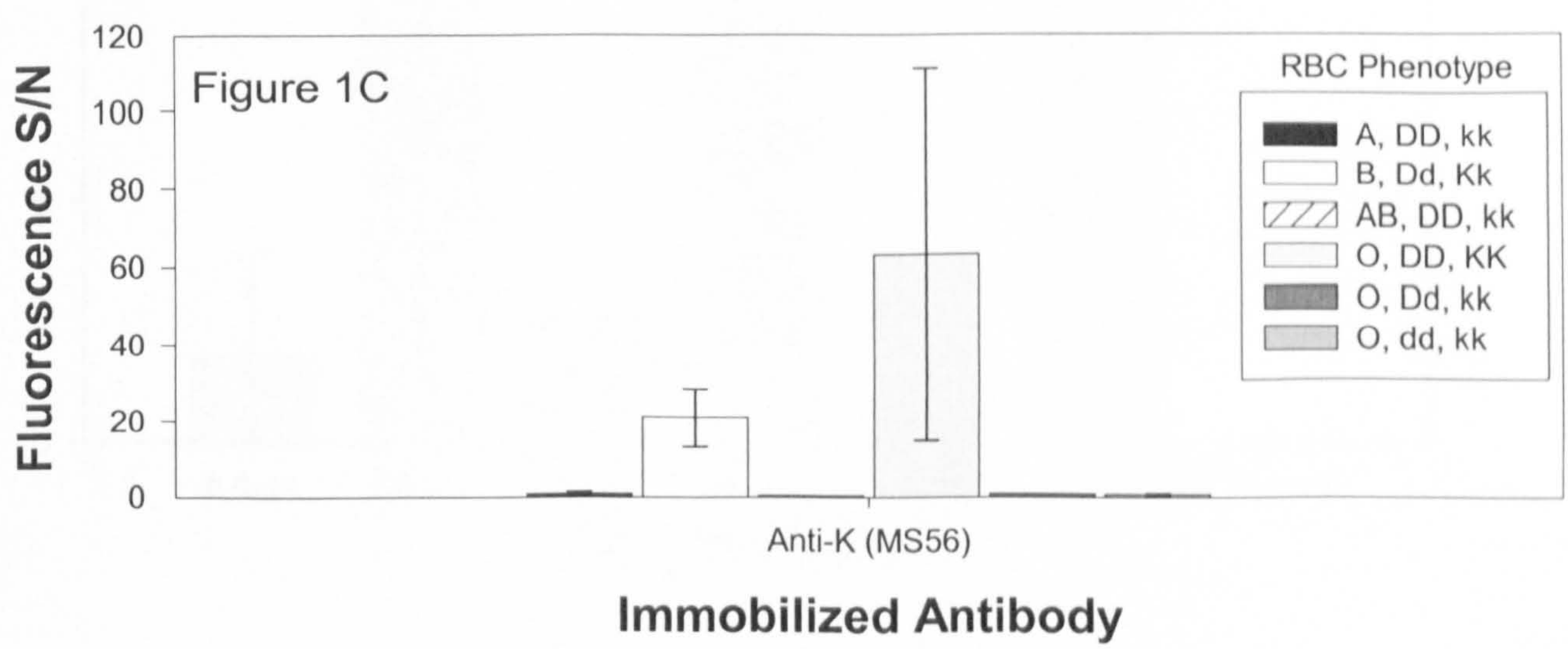

**Immobilized Antibody**

Figure 1B




**Immobilized Antibody**

Figure 1C




**Immobilized Antibody**

Figure 2A

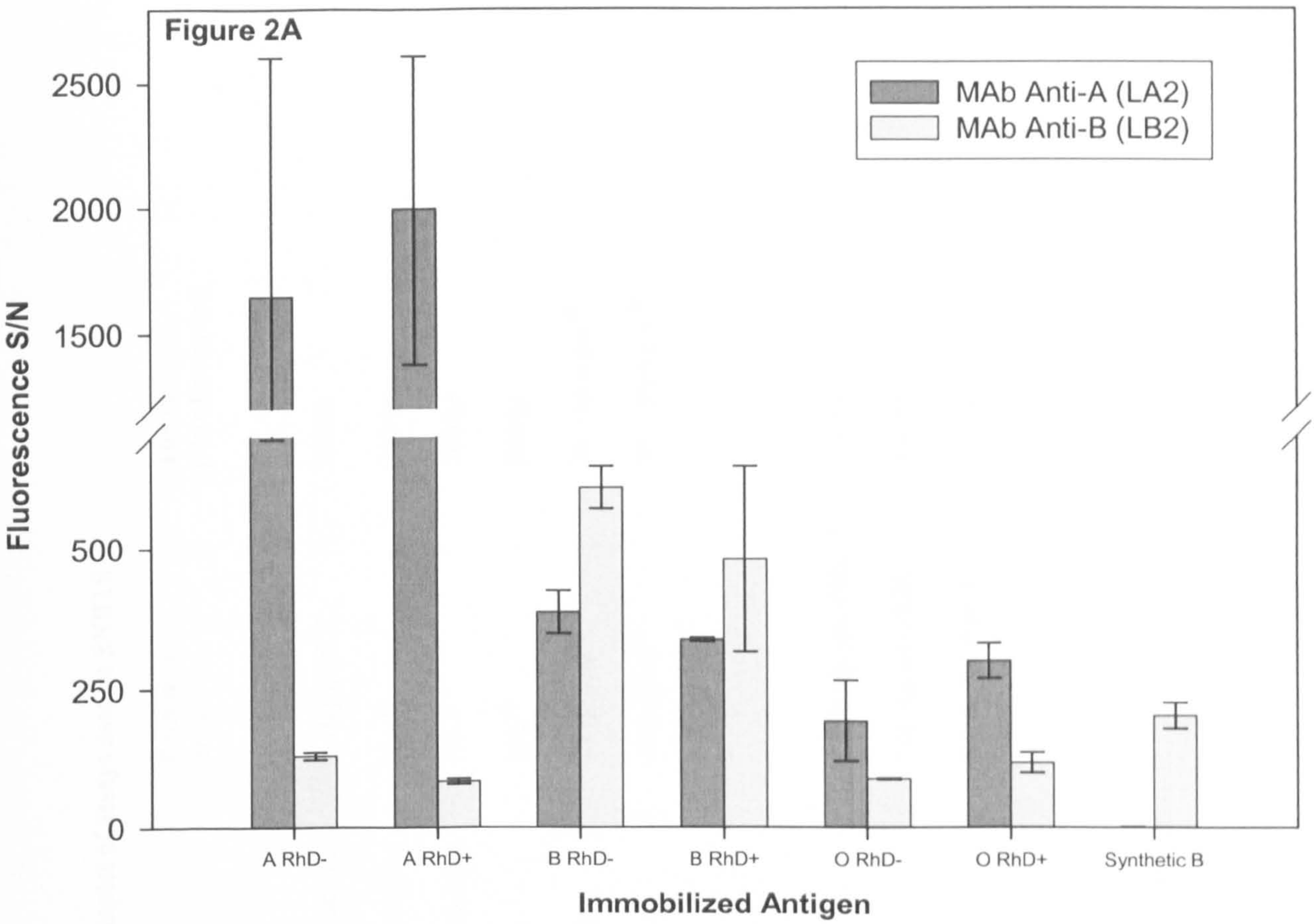



Figure 2B

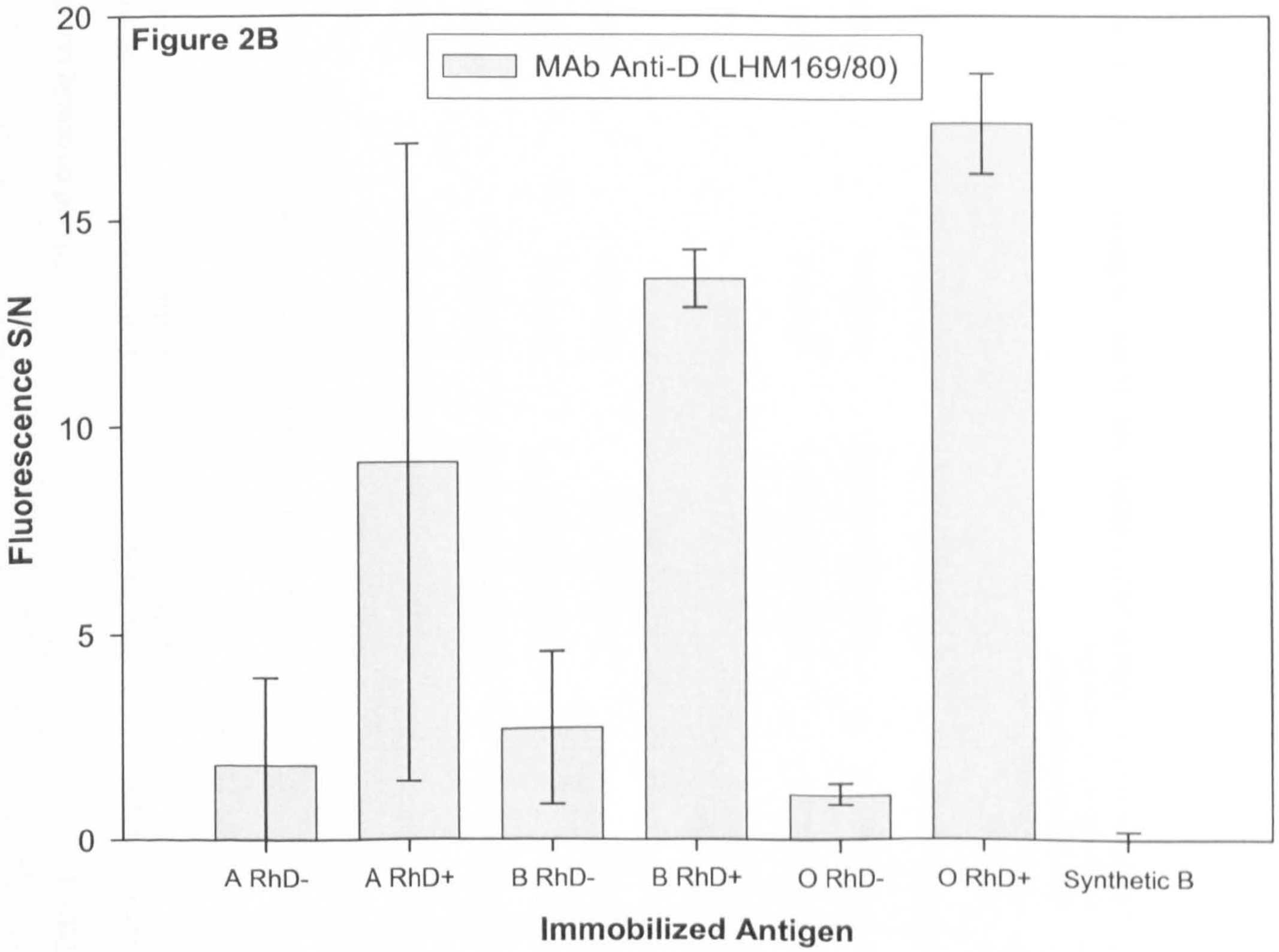


Table 1 **Blood screening assays: Examples of assays used in SNBTS**

| **Detected target** | **Detection method** | **Instrumental platform** | **Signal readout** | **Detection limit** | **Throughput (No of samples)** |
|---|---|---|---|---|---|
| PATHOGEN TESTING | | | | | |
| HBsAg | Sandwich immunoassay | Abbott Prism | Chemiluminiscence | 1-10 pg/ml [a)] | 200/hr |
| Anti-HCV | Sandwich immunoassay | Abbott Prism | Chemiluminiscence | NA [b)] | 200/hr |
| Anti-HIV1,2 | Sandwich immunoassay | Abbott Prism | Chemiluminiscence | NA [b)] | 200/hr |
| Anti-treponema | Heamagglutination | Olympus | CCD | NA [b)] | 240/hr |
| HCV RNA | Real time PCR | In-house | Fluorescence | 29 geq/ml [c)] | 30 –50/6 hrs [d)] |
| HIV1 RNA | Real time PCR | In-house | Fluorescence | 34 geq/ml [c)] | 30 – 50/6 hrs [d)] |
| BLOOD GROUP SEROLOGY | | | | | |
| ABO antigens | Heamagglutination | Olympus | CCD | 1,400 Ag sites/RBC [e)] | 240/hr |
| RhD antigen | Haemagglutination | Olympus | CCD | 200 Ag sites/RBC [e)] | 240/hr |
| Antibody screen | Heamagglutination | Olympus | CCD | 0.5 IU/ml Anti-D | 240/hr |

a) Estimate
b) Not applicable
c) geq: genome equivalent
d) minipools of 95 samples
e) Minimum level of antigen on certain phenotypes, may be below detectable limits of the test system

Table 2. Comparison of current testing algorithm with proposed microarray based testing algorithm.

| Parameter | Testing algorithms Current | Microarray-based |
|---|---|---|
| Number of instrumental platforms | 3 - 6 | 1 |
| Number of markers per assay | 1 - 4 | n x 10 |
| Number of probes per target in screening assay | up to 5 | multiple |
| Individual detection of probes | no | yes |
| Need for repeat testing | essential | non-essential |
| Result acquisition | sequential | simultaneous |
| IT result reconciliation prior to products/components issue | essential | not necessary |
| Reagent (probe) consumption | n x ul | n x pl - nl |

Table 3: Methods, techniques and materials increasing the microarray sensitivity.

| | Label/Signal Readout | Gain* | Reference/website |
|---|---|---|---|
| Signal amplification | | | |
| RCA | Fluorescence/various | $10^3 - 10^4$ | 29,30; www1.qiagen.com/molecularstaging.aspx |
| TSA | Fluorescence/various | nx100-1000 | 31 |
| Quantum dots | Semiconductor nanoparticles/ / Fluorescence | nx10 | www.qdots.com |
| Planar waveguide | Fluorescence | 10 | 34; www.zeptosens.com |
| Dye-doped nanoparticles | Fluorescence | nx10 | 35 |
| Resonance light scattering | Metal nanoparticles | 50 | 33; www.geniconsciences.com |
| Dendrimers | Fluorescence/various | 30 | 36; www.genisphere.com |

* In relation to confocal scanning of conventional small molecule dye-labeled reagents

Table 4: Characteristics of most important blood group antigens (compiled from 43-45).

| Antigen | Blood Group System | Antigen | Determinants | Size | Number of antigenic sites per Erythrocyte | Clinical Significance* |
|---|---|---|---|---|---|---|
| H (groupO) | ABO | A, B Precursor carried on glycoshingolipid or glycoprotein | Fucose determinant | Variable (branched). Up to 60 carbohydrate residues | 1.5-2 million | H |
| A | ABO | Sugar attached to H precursor oligosaccharide chain | N-Acetylgalactosamine determinant (GalNAc) | Variable (branched). Up to 60 carbohydrate residues | A1: $8.1x10^5$ - 1.17 x $10^6$<br>A2: 1.6 - 4.4 x $10^5$ | H |
| B | ABO | Sugar attached to H precursor oligosaccharide chain | Galactose determinant (Gal) | Variable (branched). Up to 60 carbohydrate residues | 6.1 - 8.3 x $10^5$ | H |
| D | Rhesus** | Multi-pass membrane protein Protein/lipid aggregates. | 8 substitutions on extracellular loops | 30-32kDa<br>417 Aa | Common: 1-3 $x10^3$<br>Weak: $2x10^2$ - $10^4$<br>High: $7.5x 10^4$ - - 2 x $10^5$ | H |
| C, c | Rhesus** | Multi-pass membrane protein Protein/lipid aggregates. | Differences at positions 60,68,103,16 of RhCE polypeptide. | 30-32kDa<br>417 Aa | ~4 x $10^4$ | M to H |
| E, e | Rhesus** | Multi-pass membrane protein Protein/lipid aggregates. | Difference at pos. 226 of RhCE polypeptide | 30-32kDa<br>417 Aa | ~2 x $10^4$ | M to H |
| Cw | Rhesus** | Multi-pass membrane protein Protein/lipid aggregates. | Difference at pos. 41 of RhCE polypeptide | 30-32kDa<br>417 Aa | 2.15 – 4 x $10^4$ | L to M |
| K, k | Kell | Single-pass membrane glycoprotein. | Difference at pos. 193 | 93kDa<br>732 Aa | 3.5 x $10^3$ - 1.8 x $10^4$ | M to H |
| Fya, Fyb | Duffy | Multi-pass membrane glycoprotein | Difference at pos. 42 | 35-43kDa<br>336 Aa | 1.35 x $10^4$ | M to H (Fya)<br>L (Fyb) |
| Jka, Jkb | Kidd | Multi-pass membrane protein | Difference at pos. 280 | 45kDa<br>391 Aa | 1.4 x $10^4$ | M to H |
| S, s | MNS | Single pass membrane protein GPB | Difference at pos. 29 | 24kDa<br>72 Aa | 2 x $10^3$ | M |
| M, N | MNS | GPA | Difference at pos. 1, 5 (O-glycans at pos.2,3&4) | 37kDa<br>131 Aa | 8 x $10^5$ | L |
| P1 | P | Globotetraosylceramide | Terminal $\alpha$-Galactose | Variable | 5 x $10^5$ | L |
| $Le^a$, $Le^b$ | Lewis | 40-100 carbohydrate chains per 300 kDa carrier molecule | Fucose ($Le^a$) or 2 Fucose ($Le^b$) attached to precursor | 300 kDa incl. carrier(average); Heterodisperse | Variable (attached by passive adsorption) | L |

* H high; M moderate; L low
**RhCE and RhD proteins are 92% identical with only 35 amino acid substitutions variance;

**A cell interaction microarray for blood phenotyping.**

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**Abstract**

Microarrays promise to revolutionise diagnostic testing since multiple assays can be performed in parallel leading to improved reliability of diagnosis. In the short term, protein microarrays have the greatest potential to impact diagnostics due to their potential for non-invasive direct sample measurements. Here, we report a protein microarray technique for selectively recognising glycan and peptide motifs on the surface of red blood cells. We present results demonstrating the efficacy of the biochip approach as a rapid, highly sensitive and specific microscale multiplex assay for blood typing. We also show that the blood chip can be used to screen erythrocyte surface antigens using whole-blood in a label-free detection mode. Finally, our results indicate this method has potential for broader applications in biochip medicine.

**Introduction**

The development of DNA microarray technology in conjunction with the completion of the Human Genome Sequence has led to great advances in the field of biomedical research.[1] Microarray technology has enabled the screening of gene activity across the entire genome of several organisms and the quality and quantity of information gained has given insights into the pathways and interactions responsible for both health and disease.[2-5] Microarrays function on the basis of molecular recognition, either between complementary DNA strands or complementary protein complexes. The fact that this recognition is specific and has high affinity means that several different molecules can be immobilised on a solid surface (probes) in order to identify and quantify their binding partners (targets) from complex mixtures in solution. The microscaling afforded by the microarray platform can also offer a number of key advantages relating to improvements in binding kinetics and increased sensitivity.[6,7]

More recently microarrays are gaining increasing interest in being used as a potential diagnostic tool for e.g. infectious disease,[8-12] markers for Rheumatoid Arthritis,[13] differentially expressed proteins in breast cancer tissue,[14] antibodies against tumor antigens,[15,16] and sets of common allergens.[17]

Protein microarrays suffer from significant complications and challenges compared to DNA microarrays. In particular, in the generation and production of protein based reagents and their inherent physico-chemical heterogeneity. While nucleic acid recognition is achieved primarily by primary sequence determinants, proteins necessitate molecular recognition through a combination of primary, secondary and tertiary structures. In addition, non-productive surface interactions can results in unfolding or disorientation preventing target binding.

The focus of this study is the development a low density protein microarray for performing the battery of tests required of all blood donations before being passed safe for transfusion. Blood type is determined by the expression of sugar (glycols) and peptide moieties on the red blood cell (erythrocyte) surface and determining the blood-type of the donation is important since transfusion of incompatible blood can be fatal.[18]

Current methods of blood donation and pre-transfusion patient testing involve haemagglutination, enzyme-linked immunosorbent assays and nucleic acid testing, which are performed on several different test platforms with varying degrees of automation. An improved system would involve robust but more rapid testing using less blood sample and reagents, perform multi-parameter tests on one miniaturised but comprehensive platform, and allow a more cost-effective test whilst ensuring compliance within the highly regulated area of blood testing diagnostics.

Two of the most commonly used blood typing techniques are microplate and column agglutination technology. Microplates of 96 stepped wells are used in which the antibody and erythrocytes are allowed to mix and incubate prior to centrifugation. Formation of an aggregated film of erythrocytes at the bottom of the plate shows a positive reaction with the antibody.[19]

The column agglutination technology method (two examples of which are the Ortho BioVue® System from Ortho Clinical Diagnostics,[20] and the DiaMed-ID Micro Typing System[21]) is currently popular for lower throughput

requirements. A gel card usually consists of six microwells each containing a 'gel' consisting either of glass beads or Sephadex. The antigen antibody reaction is allowed to take place above the gel during an incubation period before the card is centrifuged. A positive reaction product, which is demonstrated by haemagglutination, cannot pass through the spaces in the gel and therefore forms a line at the top of the gel. A negative reaction can easily pass through the spaces in the gel and this is demonstrated by the collection of erythrocytes at the bottom of the gel.
In this paper we report a robust protein microarray method for simultaneously detecting and accurately determining the type and sub-types of erythrocytes in blood. We have used this method to screen erythrocytes with complex mixtures of surface antigens and distinguish peptide from sugar antigens and also distinguished between types of peptide and sugar antigen. Remarkably, we show that microarray technique enables whole blood screening and is amenable for exploiting the intrinsic fluorescence properties of erythrocytes obviating the need for either direct or indirect labelling of samples. These results open new opportunities for the clinical use of biochips.

**Materials and Methods**

DMEM was obtained from Hyclone, UK. and hybridomas were proprietary cell lines (SNBTS). BRAD 3 was purchased from the International Blood Group Reference Laboratory, Southmead Road, Bristol. All buffers, detergents and Bovine Serum Albumin (BSA) were purchased from Sigma Aldrich. Erie Array slides were purchased from VWR, UK; Ssens Gold slides were purchased from Ssens BV, The Netherlands; Full Moon Hydrogel slides were purchased from Eurogentec, Belgium and Schott Hydrogel Slides were purchased directly from Schott UK Ltd. Poly-l-lysine slides were prepared in accordance with a previously published method.[22] Blood samples were obtained from SNBTS, Edinburgh, UK and were labelled as described using fluorescence procedure.[22]
Blood samples were obtained and used with the donors' prior consent and ethical clearance obtained from the SNBTS Human Ethics Committee.

**Microarray Experiments**

Arrays were prepared by printing onto microarray substrates (26 x 76 $mm^2$) using a Biorobotics MG II printer with 700 µm solid pins also from Biorobotics. Printed arrays were checked by microscopy to ensure that all probes had been printed. Arrays were stored under $N_2$ at < 5°C when not in use. Prior to use, the arrays were blocked in BSA. To block, the arrays were rinsed briefly in Phosphate Buffered Saline (PBS) pH 7.0 containing 1 % BSA and 0.05 % Tween 20 (submerged 10 times), then placed into a fresh container of PBS pH 7.0 containing 1 % BSA for one hour at room temperature, with constant mixing before being rinsed briefly in PBS pH 7.0 (submerged 10 times). The arrays were then centrifuged to dryness in an Eppendorf 5810R centrifuge at 1000 rpm for one minute.

**Data Acquisition and Processing**

Blood samples were contained on the array using hybridization chambers from Schleicher and Schuell (approximate capacity: 450 µl). Blood samples were incubated on arrays for 1 hour at room temperature with constant shaking. After incubation, the hybridisation chambers were removed and the slides washed in a mixture of PBS and Tween 20 (0.05 %) by vigorously submerging them ten times. The slides were then rinsed twice in PBS and centrifuged to dryness in an Eppendorf 5810R centrifuge at 1000 rpm for one minute.
Scanning was carried out using a Scanarray 5000 confocal microarray scanner from Packard Biochip Technologies (maximum laser power at slide 3 mW, excitation bandwidth 10 nm). For each array, five scans were taken using consistent pmt setting and incrementally increasing laser power settings. Microarray images were analysed with Quantarray software using the fixed circle method, and subtracting the background fluorescence from the spot value. From the five scans of each slide, the optimal scan in terms of linear range was selected on the basis of comparative scatter plot analysis.[23]
A signal to noise ratio (S/N) was calculated for each antibody spot. The noise level was determined for each slide by taking the average fluorescent intensity plus two standard deviations of the PBS spots (negative controls since no cells should be specifically bound). The signal to noise ratio was then calculated by dividing the fluorescence intensity for each spot by the noise. For each group of replicate spots, a median value was obtained. Negative controls (antibodies which do not react with erythrocytes) are also assigned S/N and shown in graphs to evaluate cross reactivity.
**Antibody purification**

# Appendix 4

Hybridomas were grown in a fermenter in DMEM/F12 media containing foetal calf serum under controlled conditions ($O_2$, $CO_2$, pH, temperature) specific for individual cell lines. After expansion of cell cultures and reaching the desired volume the antibodies were harvested using the Pellicon tangential flow filtration system (Millipore).

IgM antibodies were purified either by gel filtration or affinity chromatography. For gel filtration approximately 5 ml of culture supernatant was first filtered through a 100 kDa cut off filter to reduce its albumin content and then loaded onto a 500 ml Sephacryl S-300 column connected to a UV detector. Collected peaks were assayed for IgM activity by hemagglutination. The eluate (usually around 70 ml) was concentrated using 30 kDa centrifugation concentrators. Affinity chromatography was carried out on 2-mercaptopyridine (1 ml HiTrap IgM Purification HP Columns, Amersham Biosciences, UK) according to the manufacturer's instructions. Briefly, the column was equilibrated with 5 volumes of binding buffer (0.02 M sodium phosphate, pH 7.5; 0.8 M ammonium sulphate). Culture supernatants were adjusted to 0.8 M ammonium sulphate and subsequently filtered through a 0.45 micron filter. IgM was bound to the column by passing the sample through the column at 1 ml/min. Unbound material was washed away with 15 volumes of binding buffer and the IgM eluted with 12 volumes of elution buffer (0.02 M sodium phosphate, pH 7.5).

IgG antibodies were affinity purified on Prosep G or Prosep A (Millipore) according to the manufacturer's protocol.

Antibody profiles after purification were obtained by SDS-PAGE. Aliquots of antibody preparations were analysed on pre-cast 4-12 % NuPAGE Bis-Tris gels (Invitrogen) under reducing conditions. The gels were stained using colloidal Coomassie stain (Invitrogen) or Silver Stain Plus kit (BioRad) according to the manufacturers' instructions.

The antibody potency was measured using hemagglutination in two-fold dilutions of antibodies added to the suspension of erythrocytes. IgM agglutination is visible directly after a 5 minute incubation; IgG agglutination requires addition of anti-human globulin after an initial 20 minute sensitisation period.

## Results and Discussion

### Choice of microarray surface

Protein probe arrays are particularly sensitive to surface chemistry for attachment and retention of structural integrity and molecular recognition properties. We undertook a systematic optimisation process to identify optimal surface chemistries and tested a variety of substrates. The results of these investigations indicated that gold-coated glass slides were the preferred option with epoxysilane coated glass being the second best choice (See supplementary material, Figure S1). Since we found it interesting that gold slides gave the best S/N ratios we further investigated the difference between gold slides and epoxy silane slides. When comparing these two surfaces, there are two factors that could explain the marked difference in performance. The first is the chemistry of attachment of the antibodies to the slide and the second is the effect that the microarray surface has on the fluorophores positioned close to it. It is well established and has been extensively studied, that proximity to a noble metal surface will lead to alteration of the output of a fluorophore.[24] Although this is most pronounced for nanoparticles, it also applies to planar surfaces. To investigate this effect, we have printed fluorescently labelled biomolecules onto epoxy silane coated glass slides and gold coated glass slides. Fluorescein-labelled 25mer oligonucleotides and fluorescein-labelled red blood-cells were printed onto both epoxysilane and gold coated slides. For each combination, 200 identical spots of the fluorescently labelled biomolecule were printed on the slide. All the slides were scanned using a commercial scanner at a laser power of 75% and gain setting chosen at which a minimum of spots were saturated in order to enable comparison of intensities at a given scanner setting: for oligonucleotides 50% and for red blood cells 75%.

From Figure 1 it can be seen that while fluorescent oligonucleotides spotted on gold slides have lower fluorescence than on epoxy silane slides (median intensities 4487 and 27030 Relative fluorescent units (RFU) respectively), the intensity of spots of labelled red blood cells is comparatively higher on gold than on epoxy slides (median intensities 12126 and 4332 RFU respectively). This can be explained in terms of the enhanced electromagnetic field created by a surface plasmon wave (SPW) propagating along the gold interface. Within *ca.* 7 nm of the gold surface, non-radiative energy transfer occurs between the excited fluorophore and the surface.[25,26,27] This results in quenching of the emitted light and a concomitant decrease in the fluorescent signal associated with a spot as seen in Figure 1a, this property has, for example, been used to good effect in the design of "molecular beacons".[28] While fluorescent oligonucleotides are nanometres in size and experience a significant quenching, red blood-cells are roughly 6-8 µm in diameter and 1 µm in depth meaning that 98% of the cell volume is outside this area and the fluorescence is not significantly quenched. However, when we compare the fluorescence of red blood cells spotted on gold slides with red blood cells on epoxy silane slides (Figure 1b), the fluorescence on the gold slides is higher. At length scales greater than 7 nm and less than 100 nm, the SPW enhances the emission of fluorophores.[27,29] While the power of

the SPW is dependant on the angle at which the laser strikes the gold surface, there appears to be some enhancement even when using a non-optimized scanner, a similar effect has been shown using slides printed with a grating pattern.[30] The enhanced fluorescence caused by the excitation of red blood-cells by a surface-confined wave causes the signal from spots of blood on gold to emit a higher intensity of light than on the epoxy-silane coated films. This is a significant advantage of the use of gold as a microarray surface. Since the difference between fluorescence quenching and enhancement of signal is caused by a distance dependence, gold is an ideal surface to work with for a range of assays.[27] Gold can be easily functionalised using well established techniques for self assembled monolayer formation,[31] meaning that the distance between a fluorophore and the gold surface can be tuned by (for example) the length of an alkyl chain[26, 32] and the surface chemistry for attachment can be easily controlled by the choice of end group. This approach means that any fluorescent labels used in an assay could potentially be positioned to take advantage of the enhancement and avoid quenching. To take full advantage of this process the surface roughness of the gold may need to be optimized since this will affect the local field enhancement and the configuration of the microarray scanner should be matched to the surface plasmon resonance angle.

**Detection of cell surface glycolic groups : Typing blood group A,B and O.**

Having optimised several factors of array fabrication and assay protocol, we used this system to differentiate between several different phenotypes of erythrocyte. Blood group antigens are composed from a family of glycolipids or glycoproteins expressed at the surface of erythocytes. The ABO system is defined by polymorphisms of complex carbohydrate structures of glycoproteins and glycolipids expressed on the cell surface of erythrocytes. The basic unit of the ABO system is the O antigen which is a pentasaccharide terminated in a fucose. The A and B antigens are derivatives of this unit, varying by a single sugar residue branched from the penultimate galactose of the O antigen. In the case of A the sugar is N-acetyl galactose and in the case of B it is galactose.[33] The antibodies that characterise ABO blood types therefore differentiate on the basis of single sugar changes on the basic pentameric motif.

Using reagents that have been shown to accurately type erythrocytes in solution phase assays,[34] we printed a microarray (including a negative control antibody which we know to have no solid-phase reactivity with red blood cell antigens and buffer spots to which no red blood cells should bind) for determining blood type (the antibody details are shown in table 1). We used both gold coated and epoxy-silane coated slides in order to further establish the difference in these surfaces for this assay. It can be seen from Figure 2a that type $A_1$ cells have a strong interaction with anti-A antibodies printed on gold slides: ES15 (S/N 232), LA2 (S/N 125) and DAM1 (S/N 177); there is very little cross reactivity with other antibodies, the strongest being LB2 (S/N 2.5). For type B cells, as can be seen in Figure 2b, there is a strong reaction with LB2 (S/N 223) and a weaker reactivity with ES15 (S/N 21) as would be expected. The cross reactivity is low across the rest of the array, the strongest reaction being with LA2 (S/N 2). Type $A_2$ cells (Figure 2c) have fewer surface antigens (approximately 250,000 compared to approximately 1 million for $A_1$) but the antibody reactivities of ES15 (S/N 291), LA2 (S/N 30) and DAM1 (S/N 179) are of a similar magnitude, there is also low cross-reactivity with LB2 (S/N 1.7). The similarity of the signal reflects that the assay is not optimized to differentiate surface antigen concentrations of this order. However from figure 3f, it can be seen that type $A_x$ which has approximately 1000 surface antigens per cell has a significantly lower reactivity with the same group of antibodies on gold: ES15 (S/N 125), LA2 (S/N 3.4) and DAM1 (S/N 3.6); and maintains low cross reactivity with LB2 (S/N 2). Type O blood (Figure 2d) which has neither A or B antigens shows very low reactivity with all of the antibodies in the array, never having a S/N higher than 2 when using a gold surface, and not higher than 2.4 when using epoxy silane coated slides. In summary we have shown that erythrocytes expressing A and B antigens react specifically with their respective antisera in an array format, and express low cross reactivity with other non-specific antibodies.

For cells which have mixtures of antigens seen in Figure 3, our arrays have successfully identified both antigens simultaneously in those blood groups which have both A and B surface antigens. Type $A_1$BRhD (Figure 3a) reacts with all antibodies with S/N over 100 in all cases except our negative control (JLDM3 S/N 1). Type A2BRhD (Figure 3b) also reacts with all antibodies except JLDM3 (S/N 6) with S/N over over 100 in all cases. This shows that our array can identify complex mixtures of sugar and peptide antigens on cell surfaces.

**Detection of peptide moieties, RhD, C and E antigens**

## Appendix 4

In addition to sugar antigens, red blood cells contain important peptide antigens. The rhesus antigen which can be so important in antenatal testing is among this group of antigens.[18,35] The most common Rhesus antigens are D, C or c and E or e which are encoded on two structurally homologous genes on the Rh locus. Both antigens are 417 amino acid multi-pass integral membrane proteins with 6 exposed peptide loops on the erythrocyte surface which present discrete antigenic epitopes. The two proteins differ by 36 amino acids and the antigenic regions differ by as little as one amino acid between RhD and RhCE.[36]

From figure 3 it is clear that we can detect peptide antigens both in the presence and absence of sugar antigens. The detection of the D antigen can be seen from figure 3e since ORhD positive blood carries D antigens and neither A nor B antigens. In this case, we see a specific reaction with BRAD3 (S/N 136) with very low cross reactivity across the rest of the array. In cases where we have a mixture of sugar and peptide antigens both can be detected. For example in the case of BRhD positive cells (Figure 3c), the cells react with LB2 (S/N 363) and BRAD3 (S/N 275) while the highest non-specific cross reactivity is seen with DAM1 (S/N 4.6). Similarly type $A_1$RhD positive (Figure 3d) reacts with the same antibodies and to a similar degree as type $A_1$: ES15 (S/N 236), LA2 (S/N 55) and DAM1 (S/N 182), as well as reacting with BRAD3 (S/N 152). As mentioned above, type $A_x$RhD positive blood can be detected even though the number of sugar antigens is very low in comparison to other blood types. Types $A_1$BRhD positive and $A_2$BRhD positive (Figures 4a and b respectively) have the most complex mixture of antigens and in both cases the Rhesus antigen is reliably detected by BRAD3 (S/N 120 and 137 respectively). In all cases Gold coated slides proved to be superior to epoxy coated slides, confirming the result of the initial surface scoping exercise.

Although figure 3 shows the possibility of detecting a peptide antigen in the presence of a complex mixture of sugar antigens, it is not generally sufficient for all blood typing procedures, to only detect one type of peptide antigen. To ensure that we can detect peptide antigens and differentiate between types of peptide antigens, we have screened a panel of antibodies against rhesus antigens and used two types of red blood cell to measure the reactivity of these antibodies against different peptide surface antigens. The two blood types are: $R_1R_1$ which has only the D antigen and r''r'' which has c and E antigens but no D antigen. By measuring the reactivity of these different mixtures of surface antigen against our panel of antibodies we see from Figure 4 that the D antigen is particularly reactive with LHM59/19 (S/N 165), LHM169/80 (S/N 206) and BRAD3 (S/N 103) as well as being more weakly reactive with LHM76/58 (S/N 15), LHM76/59 (S/N 17), ESD1 (S/N 41) and LHM76/55 (S/N 15). Noticeably, there is no cross reactivity with the Anti-c (H48) or Anti E (DEM1) antibodies in the array (S/N < 1 in both cases). In comparison, blood cells from group r''r'' which has c and E antigens but no D antigens shows a distinctly different pattern of reactivity. The antibodies which reacted strongly with the D antigens in $R_1R_1$ blood have negligible fluorescent signals: LHM59/19 (S/N 1.0), LHM169/80 (S/N 1.4), BRAD3 (S/N 0.9); some of those which reacted less strongly also have a low signal for the r''r'' ie. ESD1 (S/N 1.1) and LHM76/55 (S/N 0.7) and two of those which reacted weakly with $R_1R_1$ also reacted weakly with r''r'' LHM76/58 (S/N 4.4), LHM76/59 (S/N 2.5), suggesting a non-specific cross reactivity. So from this panel we can select at least three strong candidates for reliable detection of the D antigen: LHM59/19, LHM169/80 and BRAD3. Given that r''r'' has c and E antigens on its surface, we expect reactions with Anti-c and Anti-E antibodies as seen in figure 4 (H48 S/N 13 and DEM1 S/N 4.3) which compare favourably with the their $R_1R_1$ reactivity (S/N 0.5 and 0.4 respectively). This shows three antibodies from the panel screened emerge as candidates for detection of the D antigen. The Anti-c and Anti-E antibodies, while providing a good negative control in this case, also show the potential to differentiate between common peptide antigens.

**Differential detection sensitivity between attachment and solution phase antibody reagents**

We have screened a panel of antibodies for their blood grouping performance and found large differences in solid-phase microarray performance between antibodies of similar solution phase heamagglutination activity. For example, in table 1, it can be seen that NLA2, NLB2 and NES15 are very similar in their solution phase potency (i.e. they cause agglutination of cells at the same dilution), all are of the same antibody subclass and have similar purity profiles by SDS PAGE, but have widely varying reactivity when used on a microarray. Similarly, LHM76/59 and LHM 169/80 (both anti-D) have the same potency in an agglutination assay but the microarray S/N of the latter is nearly 14 times greater than that of the former. Whether these differences are due to the structural stability or the orientation of the antibody on the surface, we have not investigated, however it is clear that there are differences in antibody performance between solution and solid phase that cannot be accounted for in terms of antibody class or purity.

**Screening whole blood**

# Appendix 4

Using purified and labelled cells to type blood on a microarray requires several sample preparation and derivatisation steps that are all potential sources of variation for experimental output. In order to simplify this procedure, we attempted to type whole blood and thus dispose of some blood pre-treatment steps. To this end, we attempted to label whole blood using fluorescein isothiocyanate (FITC) and then incubate this on an array in the expectation that the fluorescently labelled red cells would be quantifiable where they had bound to antibody spots. Although red cells were clearly bound to the spots and were visible by phase contrast microscopy, when scanned using FITC settings the fluorescence from the background between the spots was so strong that it overpowered the specific signal from the spots.

This can probably be explained by considering the make up of the blood proteome. About 40% of the human serum proteome is Human Serum Albumin which will have been fluorescently labelled at the same time as the red cells. This occurs since FITC non-specifically labels all proteins. HSA is known to bind non-specifically to several proteins,[37] and in high concentrations such a relatively weak interaction can be responsible for the high background between the antibody spots. In order to circumvent this problem in whole blood typing, we examined whether the intrinsic fluorescence from erythrocytes is sufficient to quantify the binding reaction.

**Label-free cell phenotyping**

Figure 5 is a graph showing the reactivity of an array of antibodies with labelled red cells, whole blood, whole blood diluted 1:5 with PBS and whole blood diluted 1:10 with PBS. It can be seen from this graph that the pattern of reactivity is the same for labelled cells as for whole blood but that the signal intensity is lesser without labelling. However, it is clear from the data that in all cases, the signal to noise ratios are high enough to allow discrimination between type A, B and O blood. For type A cells, 1:5 diluted blood appears to have the highest S/N ratio but for B, neat whole blood has the highest S/N.

Red blood cells have an absorbance spectrum as shown in figure 6 inset. This absorbance spectrum is typical of oxy-haemoglobin, as would be expected with native red blood cells. Since we originally thought that haemoglobin species were responsible for the fluorescence of erythrocytes, we tried to maximise the fluorescent signal by tuning the excitation wavelength to the absorbance spectrum shown in figure 6 inset. Since unlabelled red blood cells absorb strongly at 420, 540 and 580 nm, we would expect one of these wavelengths to give the strongest fluorescence when excited. The peak at 420 nm has the strongest absorbance but since commercial microarray scanners do not have lasers which can excite at this wavelength, the lowest excitation available was 488 nm. We compared the signal to noise ratio for this setting with three other settings which excite at the 543 nm (close to the absorbance peak) as detailed in table 2. The signal to noise ratios obtained using these settings are shown in figure 6. After scanning, the 488/1 scan was repeated to ensure that deleterious bleaching of fluorescence could be controlled for. From this figure, we show that the optimal scanner setting is 488/1, the setting commonly used to detect fluorescein. Since this does not correlate with the absorbance spectrum of haemoglobin, we suggest that the majority of autofluorescence from the erythrocytes is caused by other cellular components such as lipids and proteins.

**Conclusion**

We have shown that we can use protein microarray technology to phenotype erythrocytes by detecting complex mixtures of antigens on cell surfaces. The antigens are both sugar antigens which tend to be well presented and easily accessible and peptide antigens which are epitopes of transmembrane proteins and therefore buried and held more closely to the cell surface. We also show that we can differentiate different types of surface antigen, both sugar and peptide, using the correct choice of antibodies. The choice of antibodies is not immediately obvious and we see that some antibodies which have very similar solution reactivity do not perform comparably on a microarray surface. Until the structural and surface characteristics that control these differences are fully understood, the best way of constructing such a protein microarray for such a purpose will be by screening a library of antibodies in the way we have described. We have found that an array consisting of LA2, LB2, ES15, DAM1, LHM169/80, LHM59/15, DEM1 and H48 is sufficient to phenotype several classes of erythrocyte. In addition we have demonstrated the particular utility of gold coated array slides for the screening of cells and attribute this in part to the interaction between the fluorophore and excited plasmons on the microarray slide surface. By maximising S/N through the correct choice of antibody and microarray surface we have created an array which can not only phenotype labelled erythrocytes, but also screen whole blood for surface antigens. This obviates both labelling and sample purification steps, significantly simplifying the blood typing procedure.

# Appendix 4

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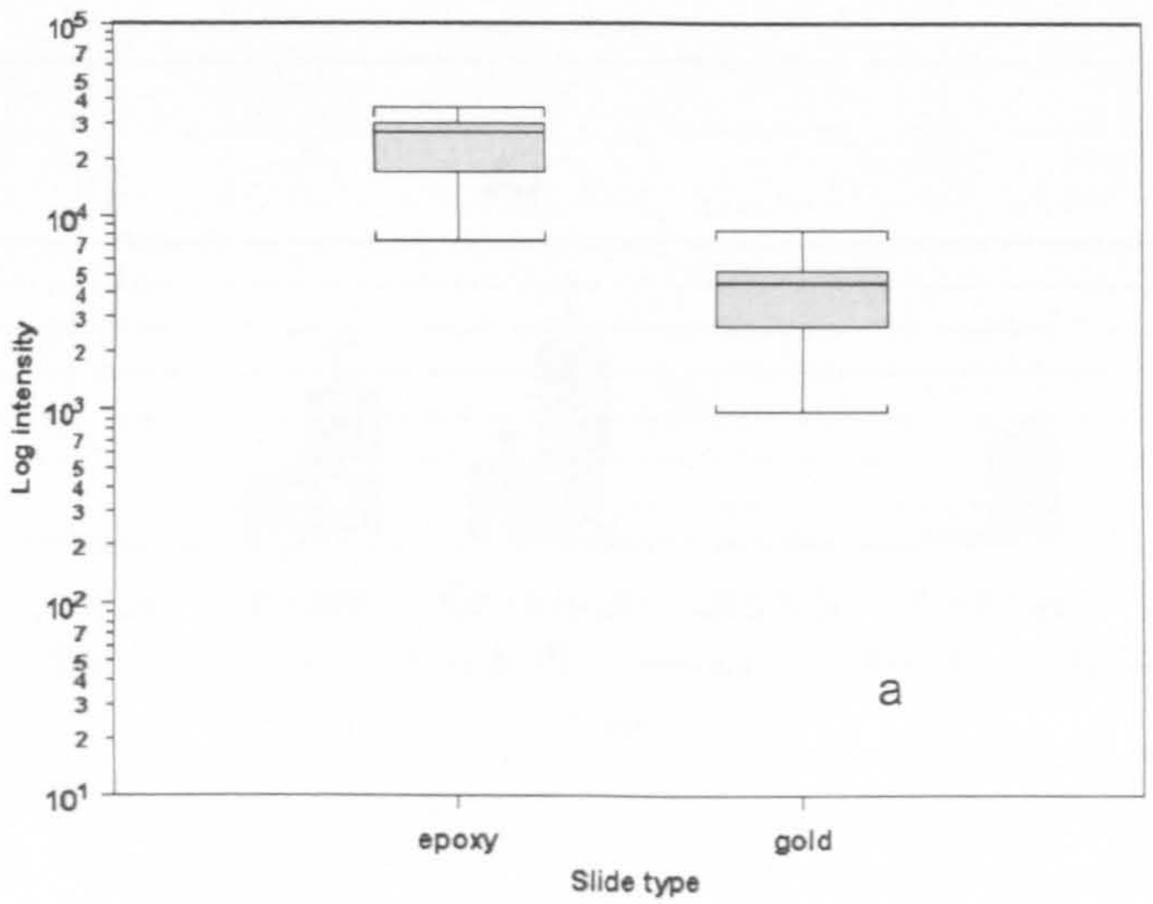
# Appendix 4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Slide type
epoxy
gold
a

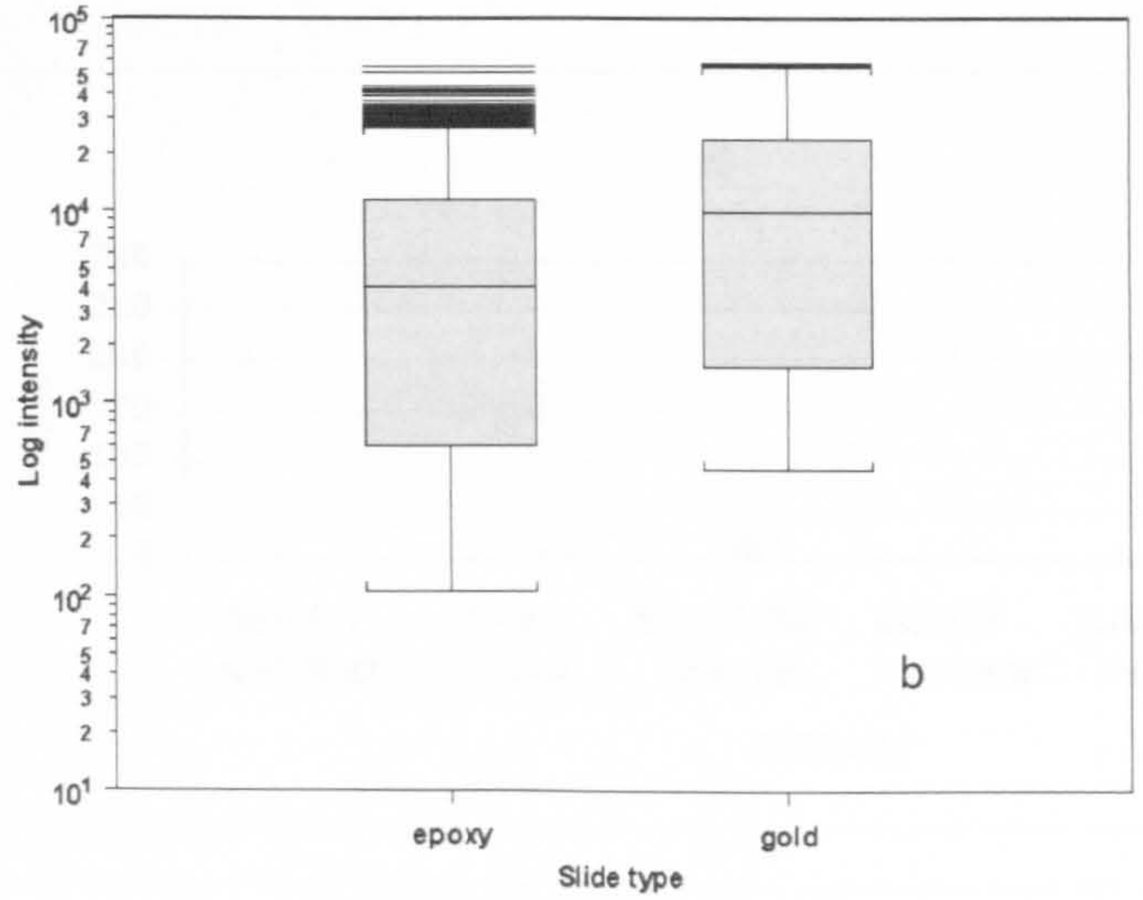
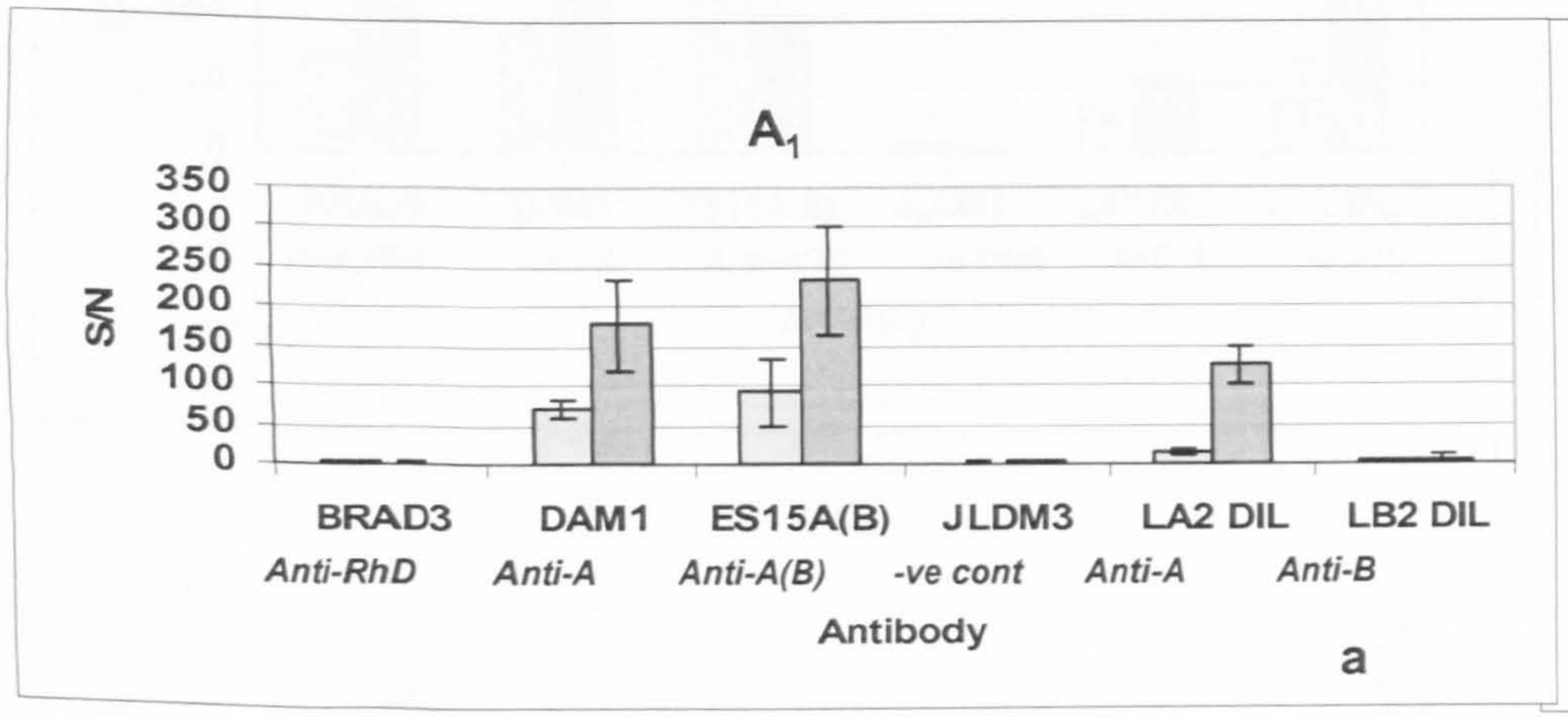
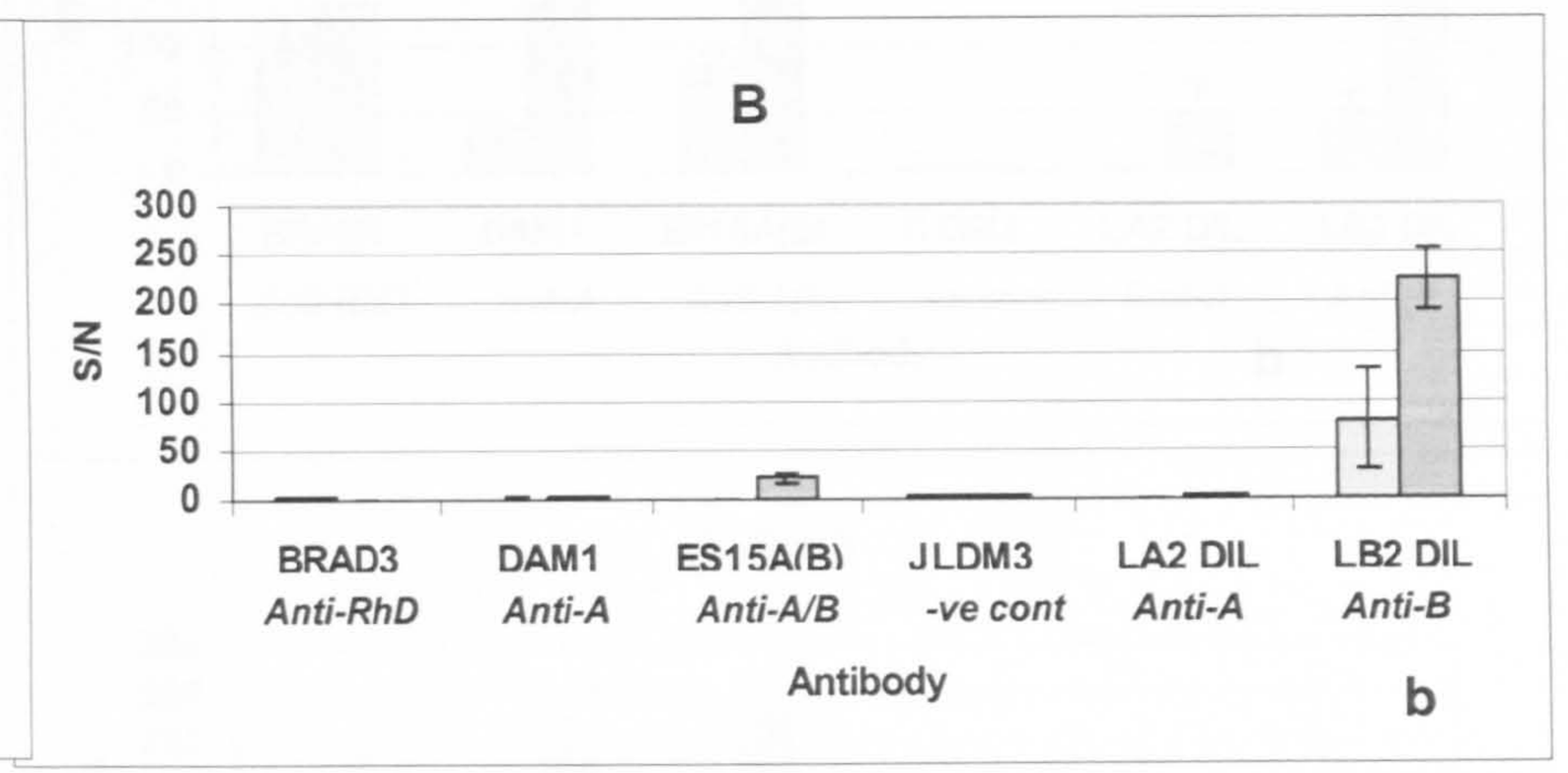
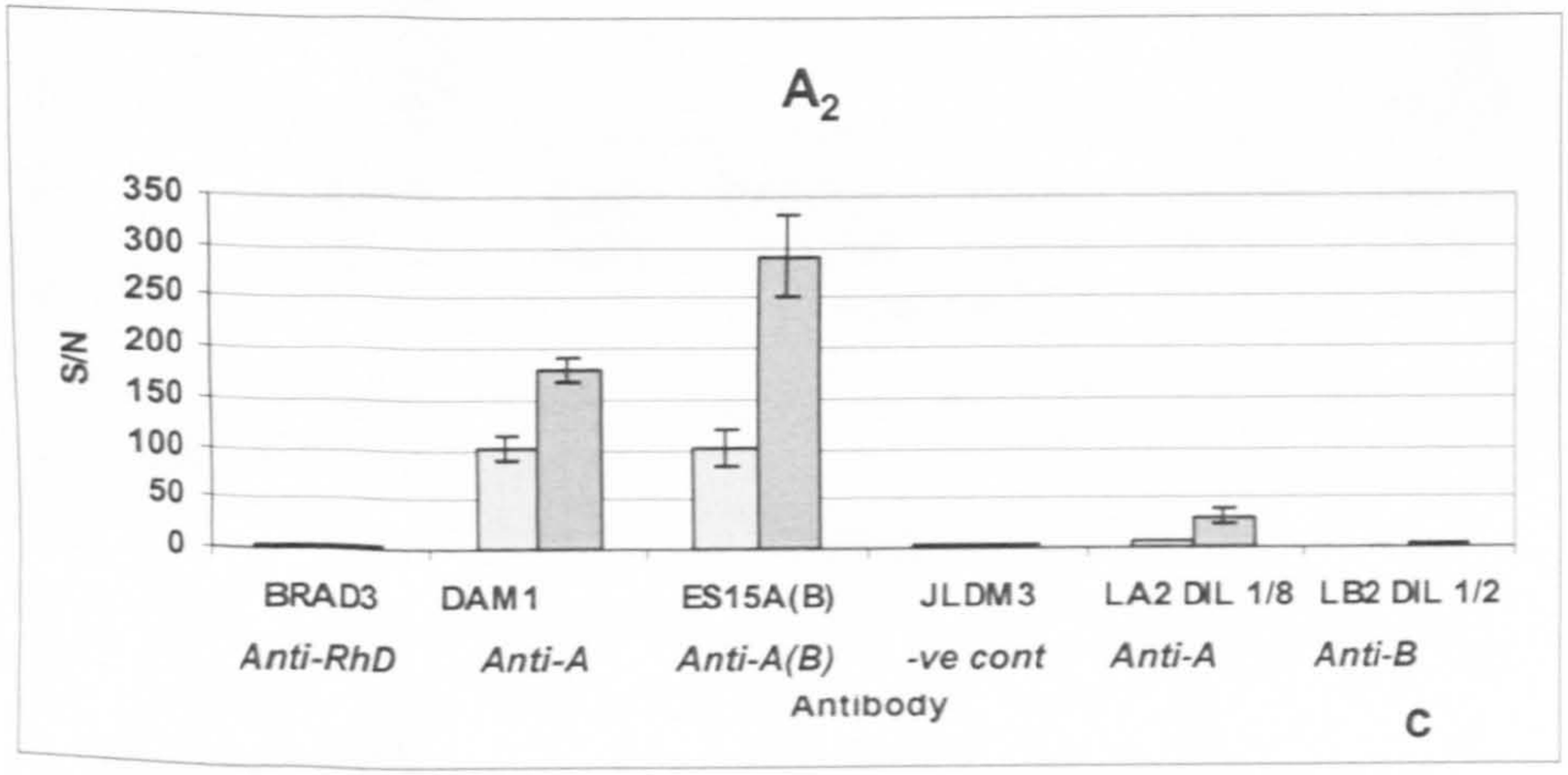

Log intensity
Slide type
epoxy
gold
b

Figure 1


A1
S/N
350
300
250
200
150
100
50
0
BRAD3
Anti-RhD
DAM1
Anti-A
ES15A(B)
Anti-A(B)
JLDM3
-ve cont
LA2 DIL
Anti-A
LB2 DIL
Anti-B
Antibody
a


B
S/N
300
250
200
150
100
50
0
BRAD3
Anti-RhD
DAM1
Anti-A
ES15A(B)
Anti-A/B
JLDM3
-ve cont
LA2 DIL
Anti-A
LB2 DIL
Anti-B
Antibody
b


A2
S/N
350
300
250
200
150
100
50
0
BRAD3
Anti-RhD
DAM1
Anti-A
ES15A(B)
Anti-A(B)
JLDM3
-ve cont
LA2 DIL 1/8
Anti-A
LB2 DIL 1/2
Anti-B
Antibody
c

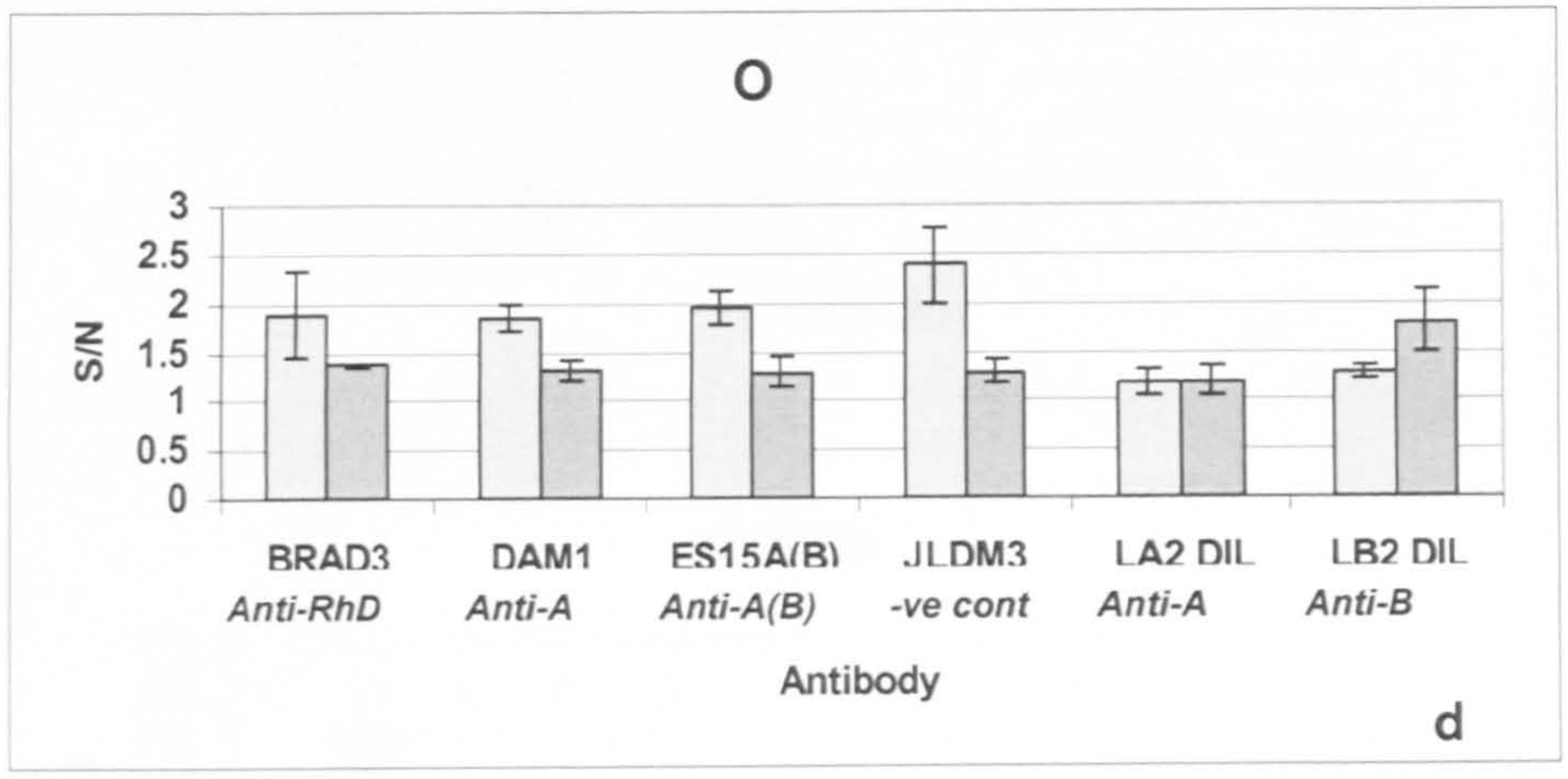
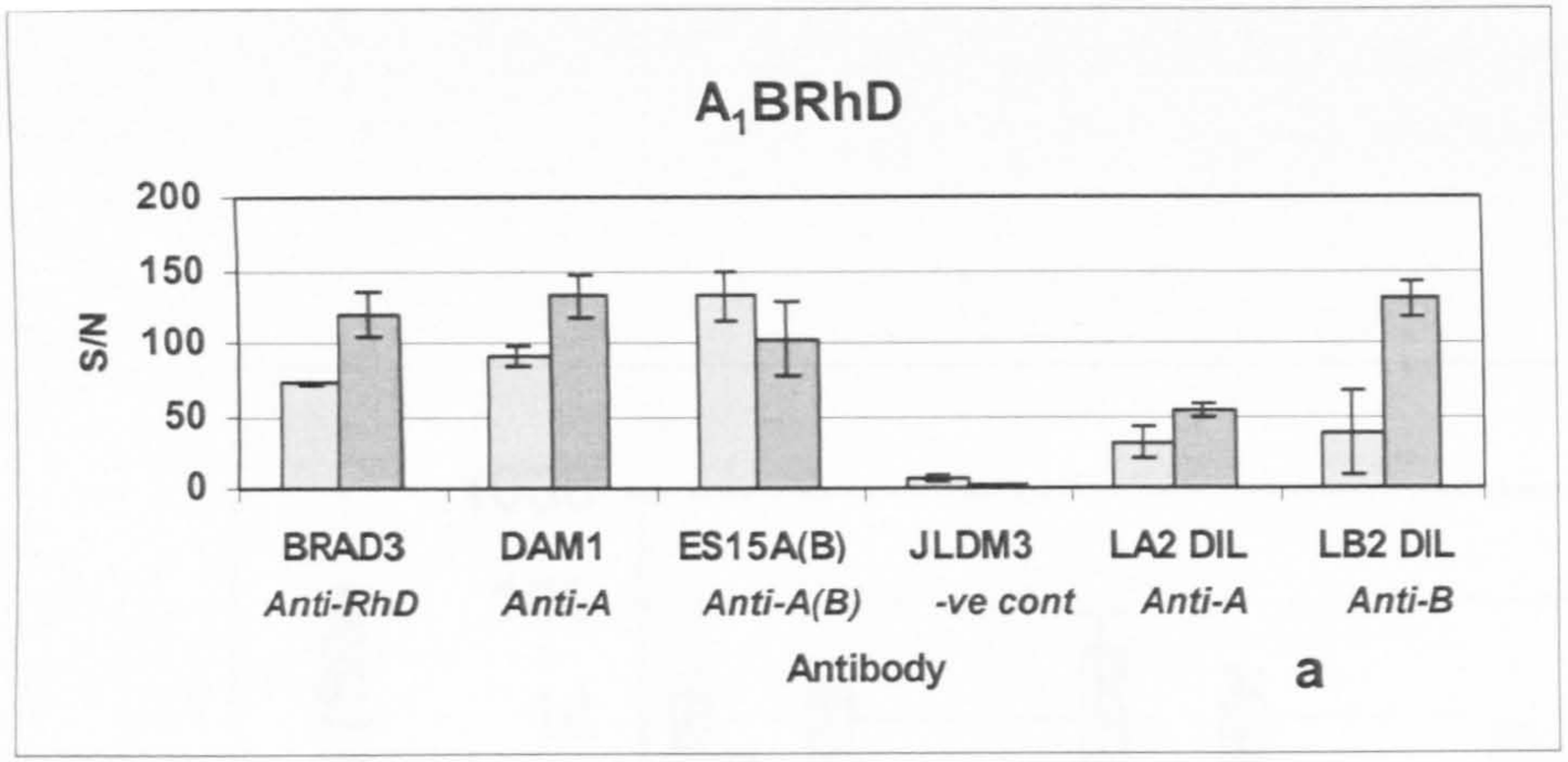
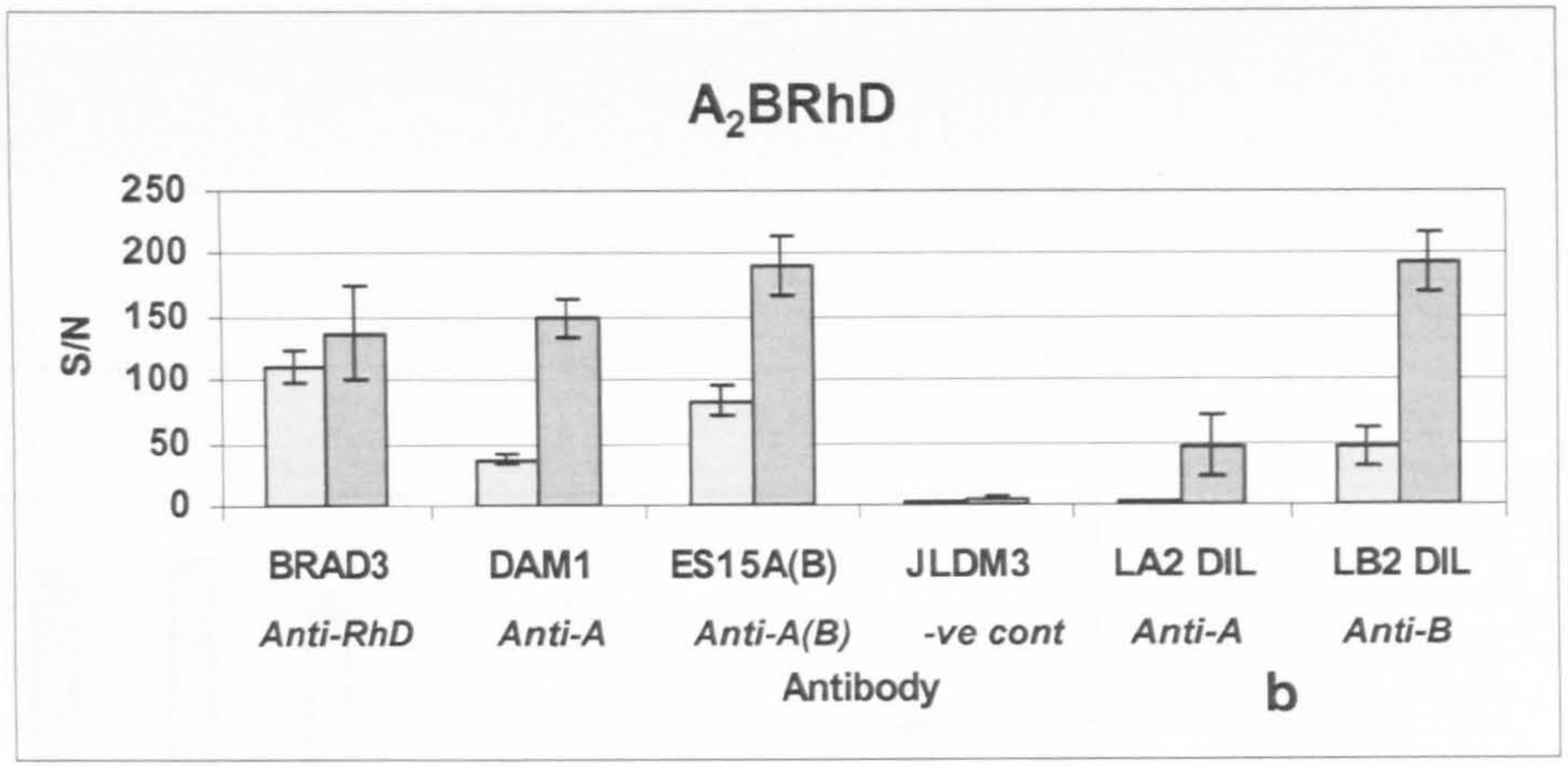
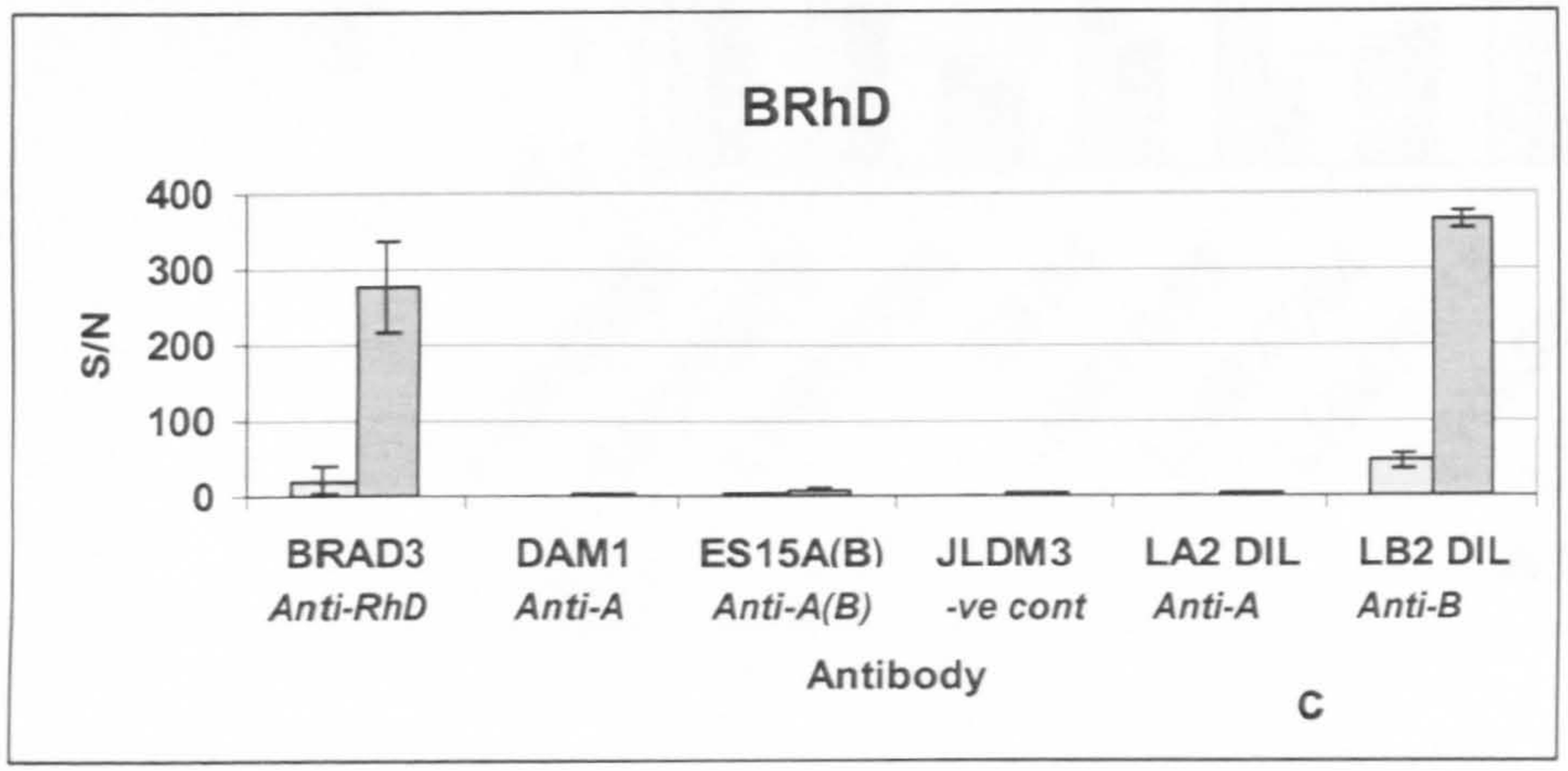
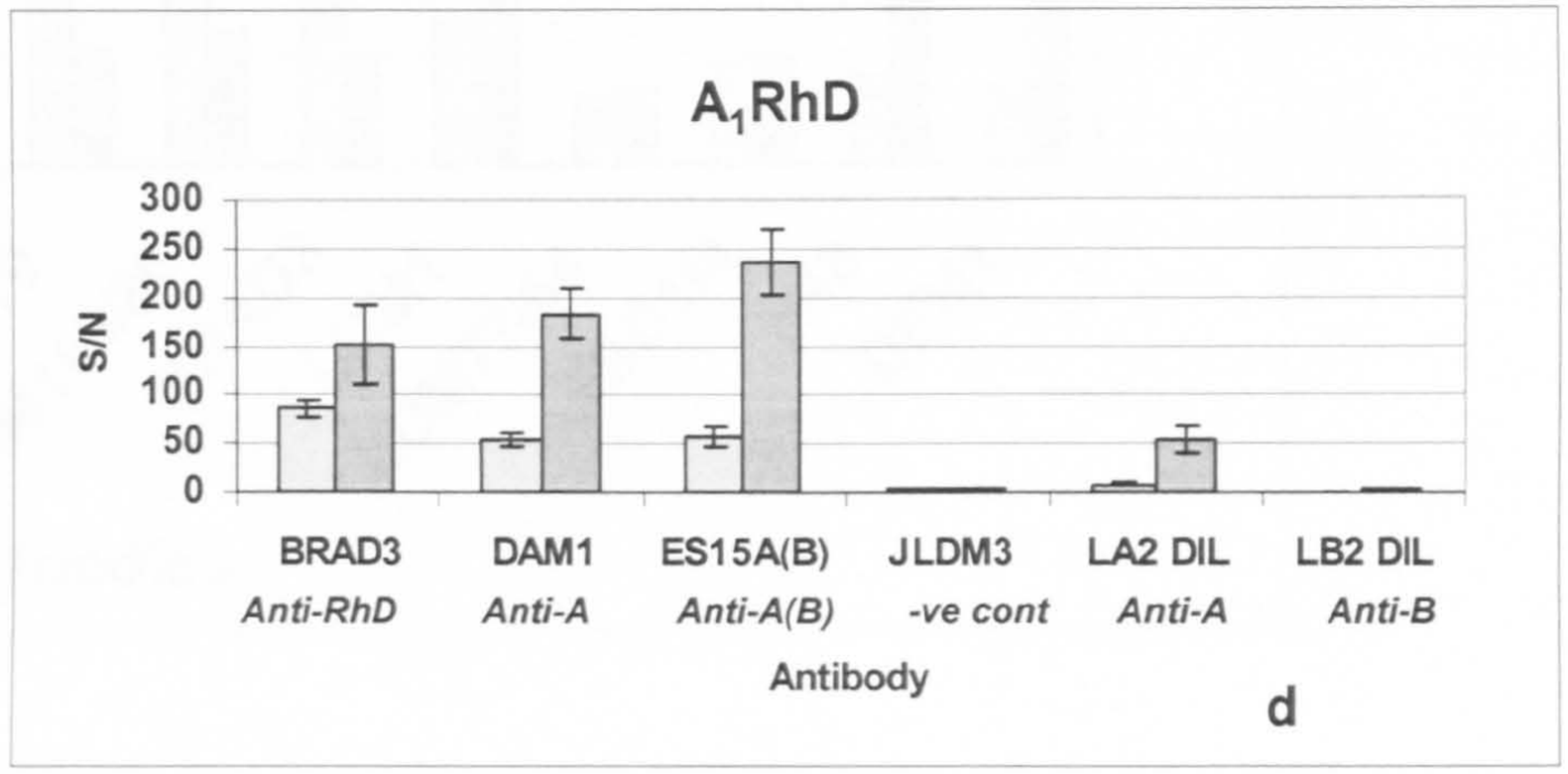

O
S/N
3
2.5
2
1.5
1
0.5
0
BRAD3
Anti-RhD
DAM1
Anti-A
ES15A(B)
Anti-A(B)
JLDM3
-ve cont
LA2 DIL
Anti-A
LB2 DIL
Anti-B
Antibody
d

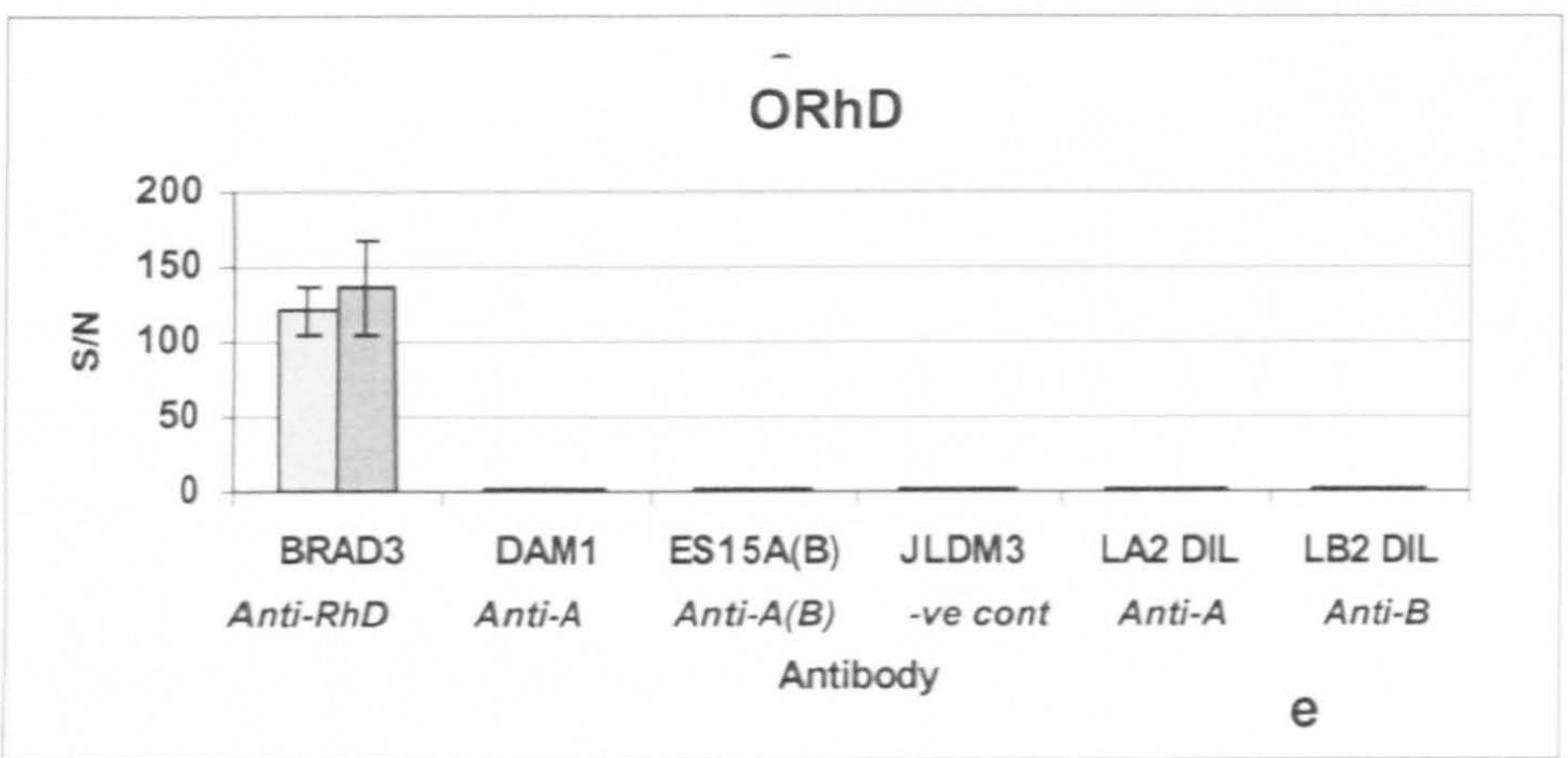
Figure 2


A1BRhD
S/N
200
150
100
50
0
BRAD3
Anti-RhD
DAM1
Anti-A
ES15A(B)
Anti-A(B)
JLDM3
-ve cont
LA2 DIL
Anti-A
LB2 DIL
Anti-B
Antibody
a


A2BRhD
S/N
250
200
150
100
50
0
BRAD3
Anti-RhD
DAM1
Anti-A
ES15A(B)
Anti-A(B)
JLDM3
-ve cont
LA2 DIL
Anti-A
LB2 DIL
Anti-B
Antibody
b


BRhD
S/N
400
300
200
100
0
BRAD3
Anti-RhD
DAM1
Anti-A
ES15A(B)
Anti-A(B)
JLDM3
-ve cont
LA2 DIL
Anti-A
LB2 DIL
Anti-B
Antibody
c


A1RhD
S/N
300
250
200
150
100
50
0
BRAD3
Anti-RhD
DAM1
Anti-A
ES15A(B)
Anti-A(B)
JLDM3
-ve cont
LA2 DIL
Anti-A
LB2 DIL
Anti-B
Antibody
d


ORhD
S/N
200
150
100
50
0
BRAD3
Anti-RhD
DAM1
Anti-A
ES15A(B)
Anti-A(B)
JLDM3
-ve cont
LA2 DIL
Anti-A
LB2 DIL
Anti-B
Antibody
e

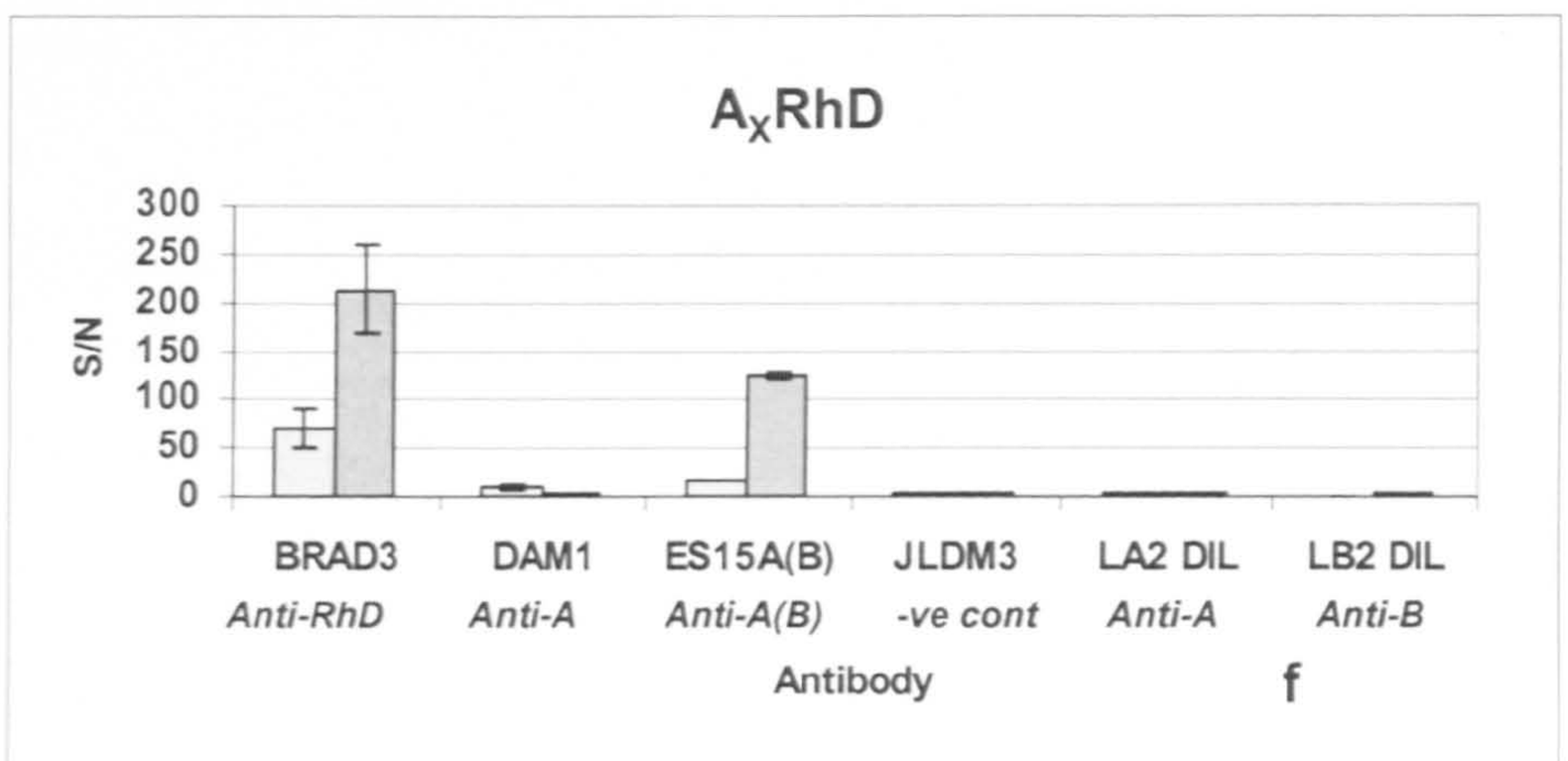

AxRhD
S/N
300
250
200
150
100
50
0
BRAD3
Anti-RhD
DAM1
Anti-A
ES15A(B)
Anti-A(B)
JLDM3
-ve cont
LA2 DIL
Anti-A
LB2 DIL
Anti-B
Antibody
f

Figure 3

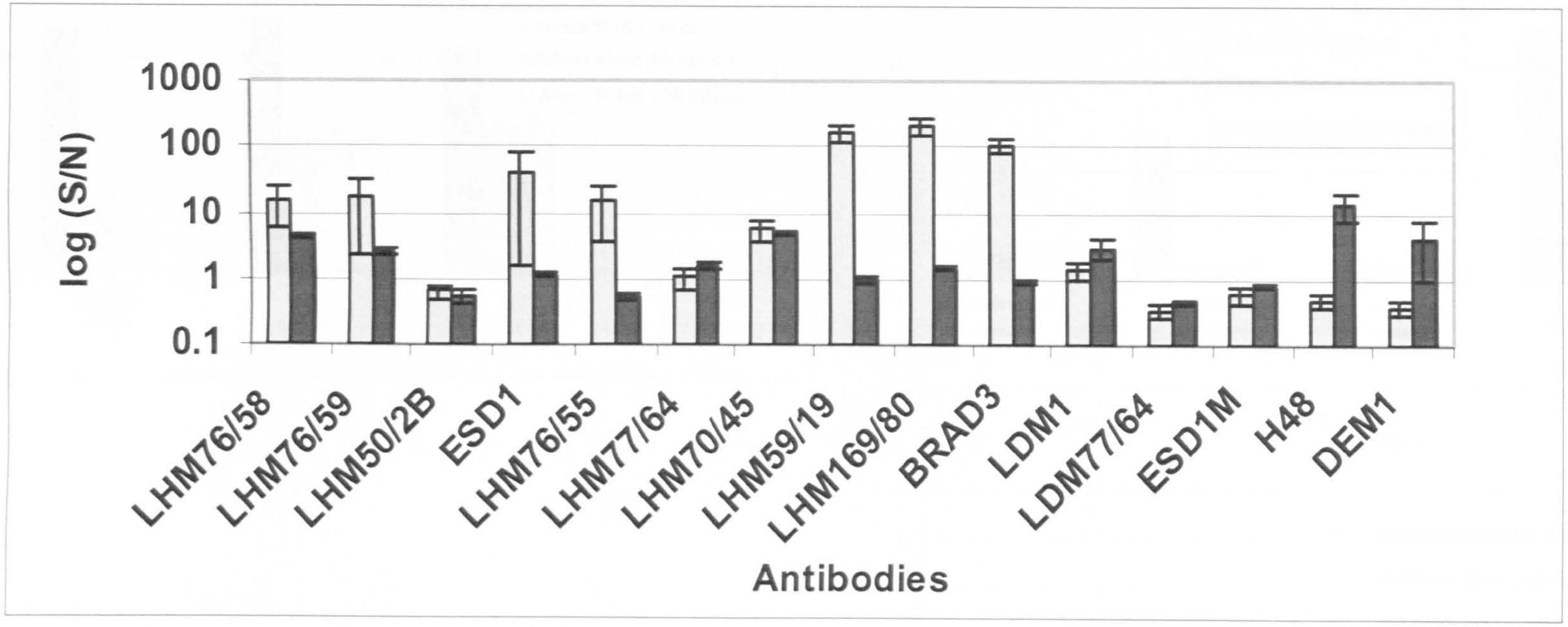
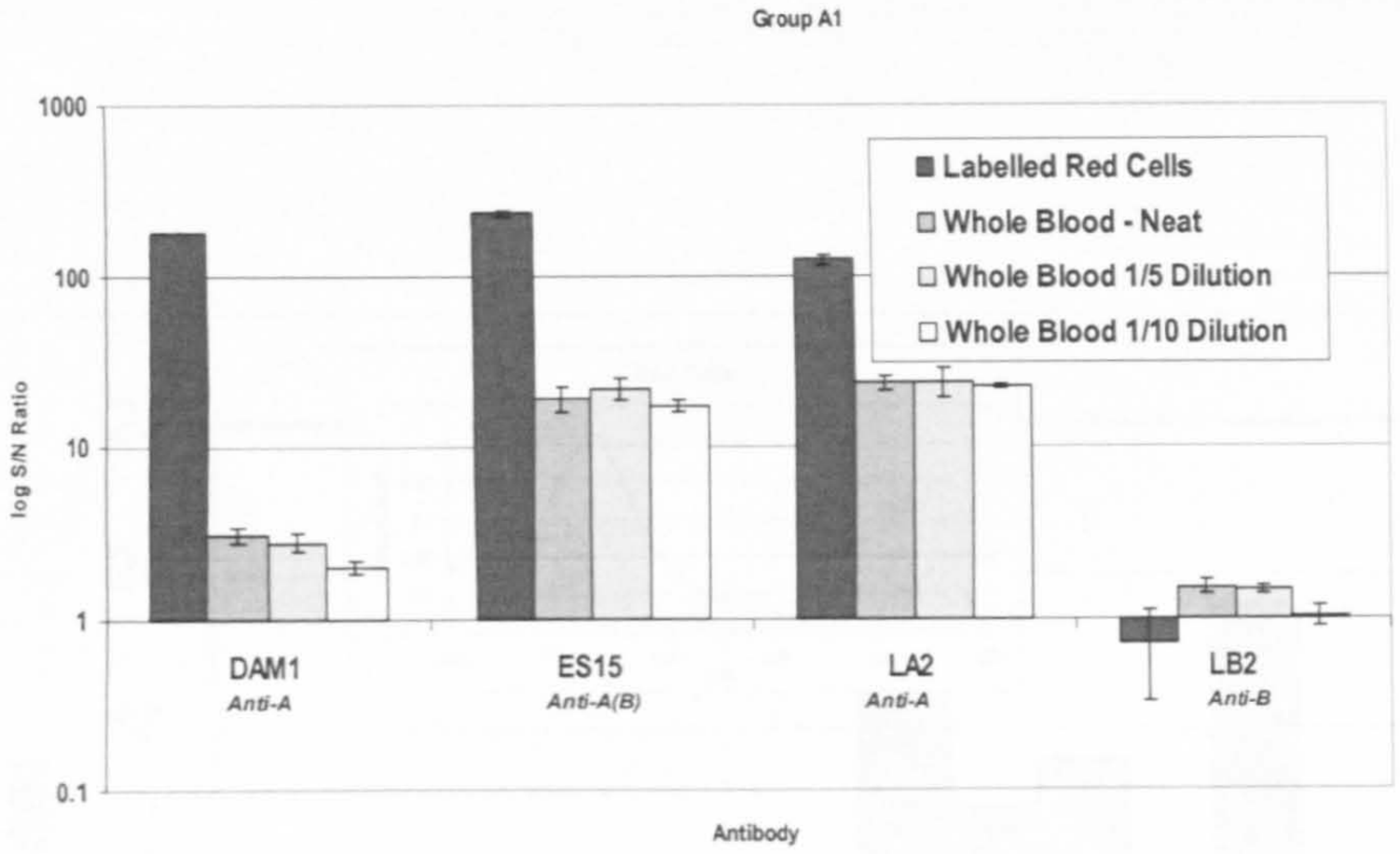
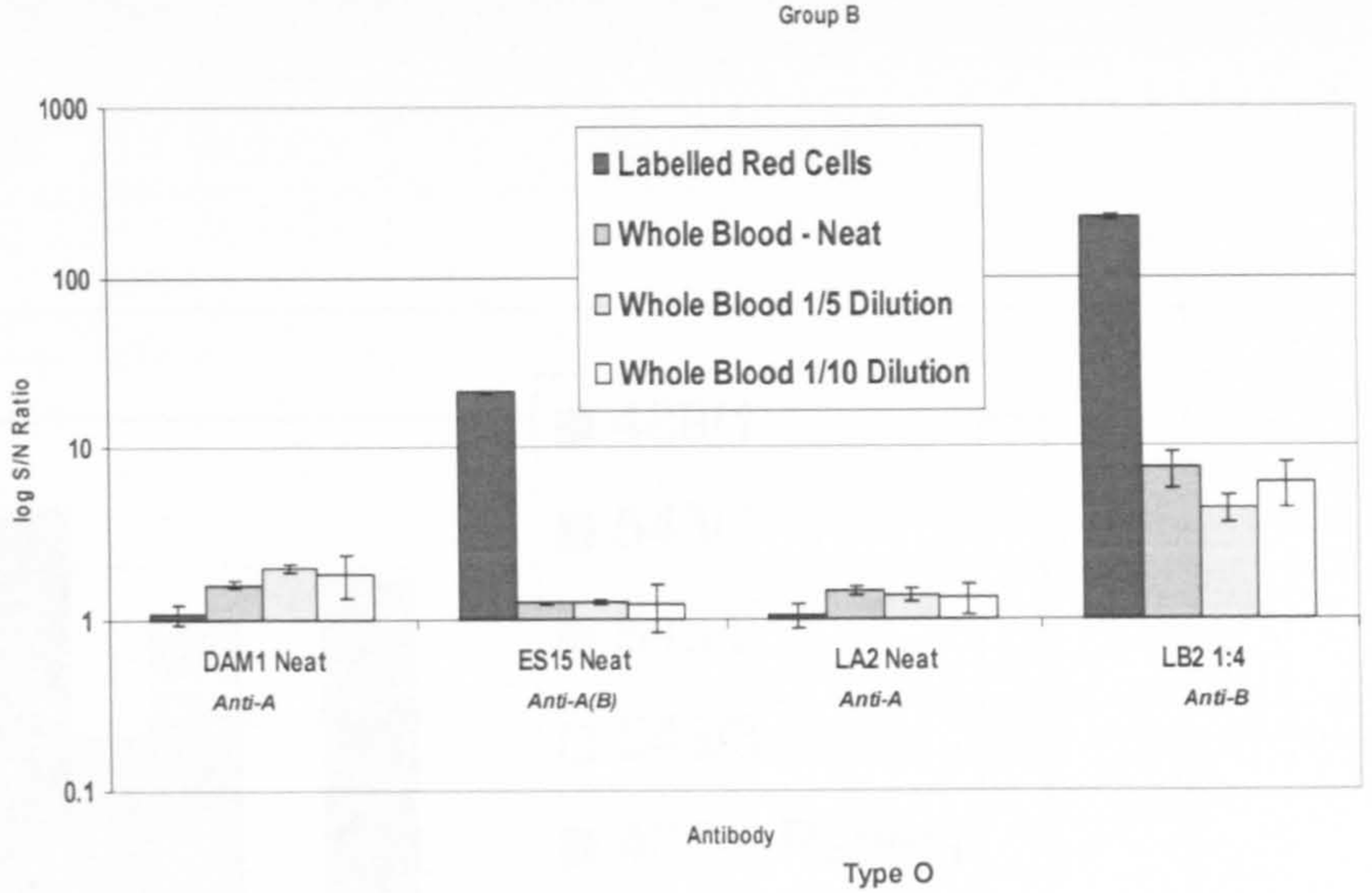

log (S/N)
1000
100
10
1
0.1
LHM76/58
LHM76/59
LHM50/2B
ESD1
LHM76/55
LHM77/64
LHM70/45
LHM59/19
LHM169/80
BRAD3
LDM1
LDM77/64
ESD1M
H48
DEM1
Antibodies

Figure 4




Type O

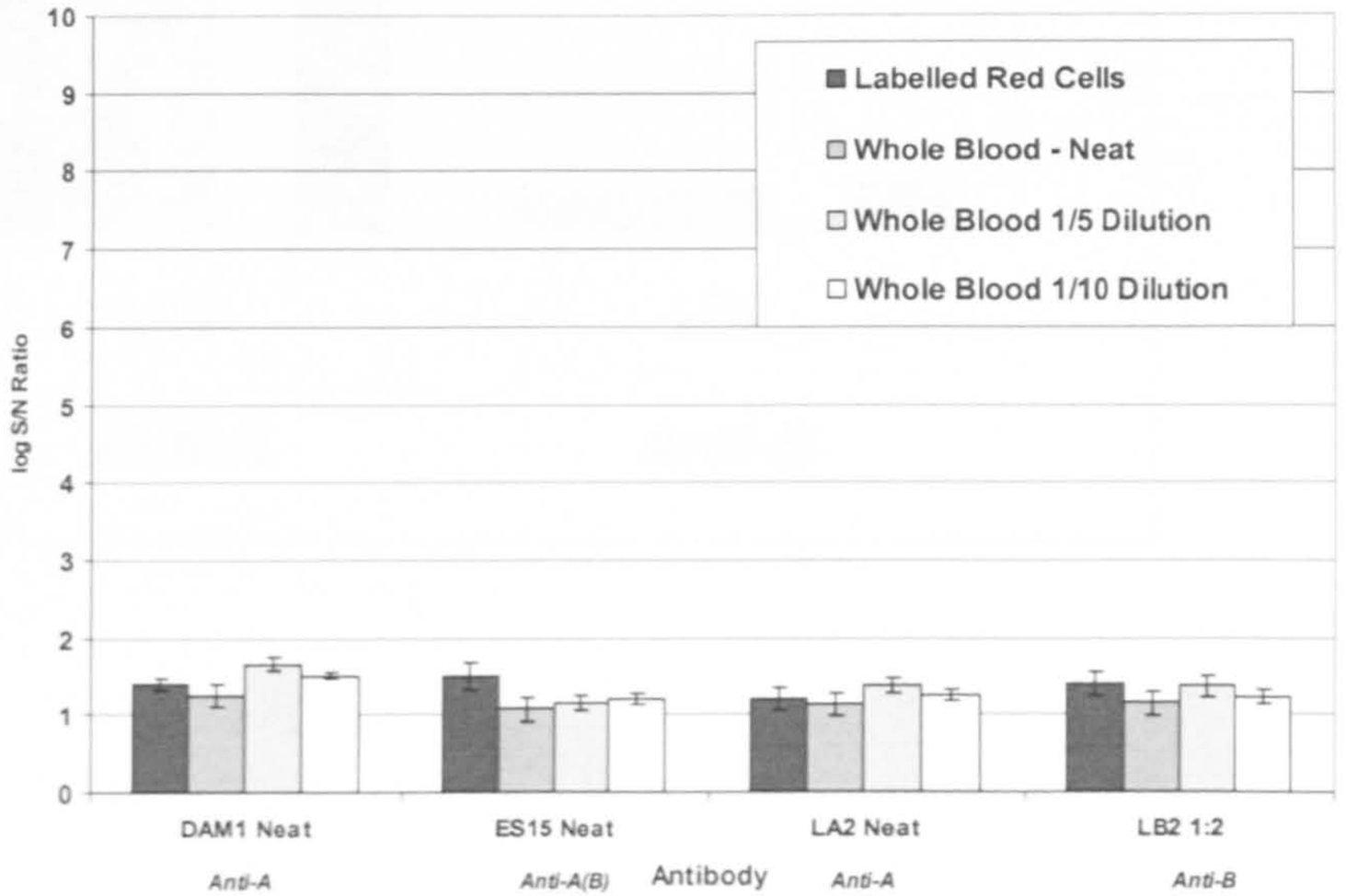



Figure 5

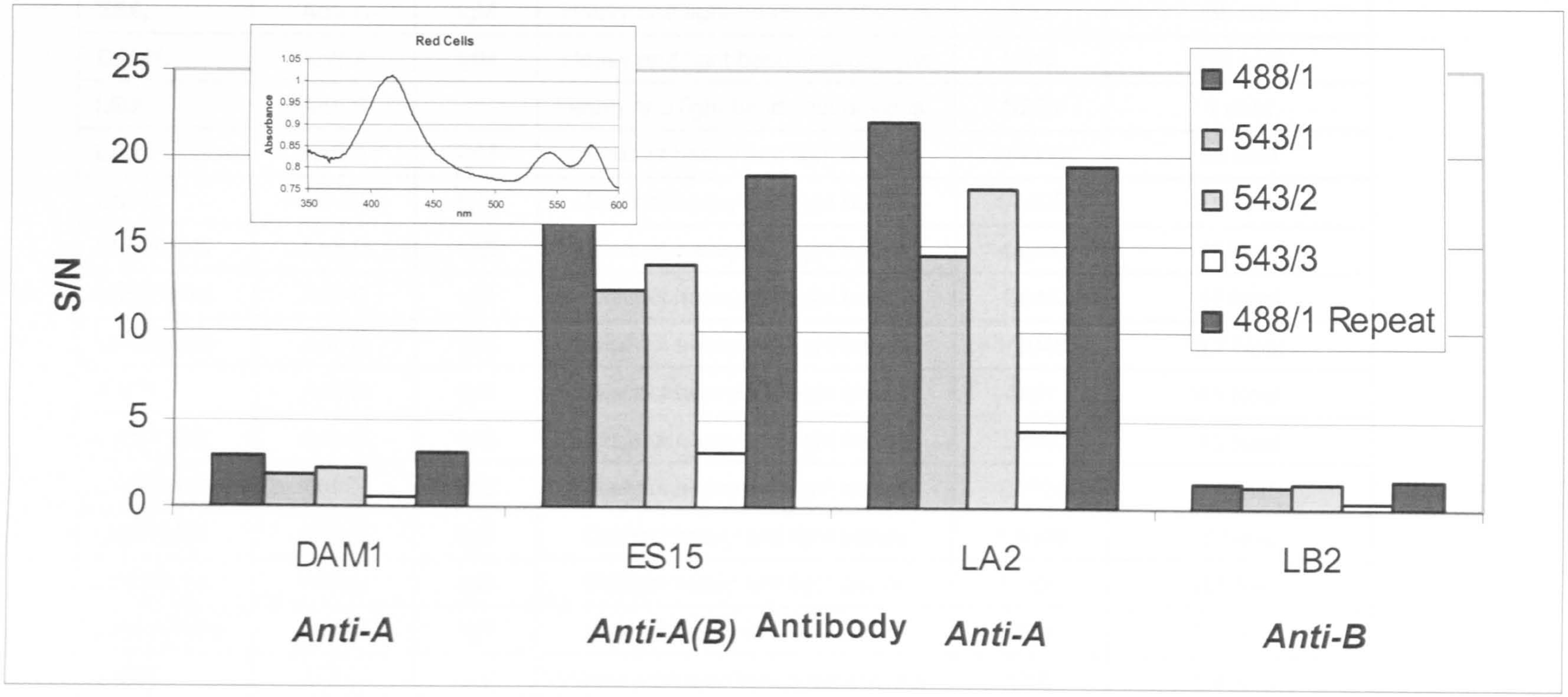

Red Cells
Absorbance
nm
S/N
25
20
15
10
5
0
488/1
543/1
543/2
543/3
488/1 Repeat
DAM1
ES15
LA2
LB2
Anti-A
Anti-A(B)
Antibody
Anti-A
Anti-B

Figure 6

| Antibody | Specificity | Ab Class | SDS PAGE | potency | Microarray S/N |
|---|---|---|---|---|---|
| **LA1** | Anti-A | IgM | Distinct heavy and light bands | 1/256 | 3.5 Neat |
| **LA2,** | Anti-A | IgM | Distinct heavy and light bands | 1/4000 | 301 at 1:8 dil |
| **ES9,** | Anti-A | IgM | Heavy and light bands not obvious | 1/32 | 258 neat |
| **DAM1,** | Anti-A | IgM | Heavy and light bands not obvious | 1/512 | 215 neat |
| **LB3** | Anti-B | | Heavy and light bands not obvious | 1/256 | 1 neat |
| **LB2,** | Anti-B | IgM | Distinct heavy and light bands | 1/4000 | 96 neat |
| **ES15,** | Anti-A(B) | IgM | Distinct heavy and light bands | 1/4000 | 301 Neat |
| **LHM76/58** | Anti-D | IgG | Distinct heavy and light bands | 1/64K | 15 Neat |
| **LHM76/59** | Anti-D | IgG | Distinct heavy and light bands | 1/64K | 17 Neat |
| **LHM50/2B** | Anti-D | IgG | Distinct heavy and light bands | 1/2048 | 0.7 Neat |
| **ESD1** | Anti-D | IgG | Distinct heavy and light bands | 1/8K | 41 Neat |
| **LHM76/55** | Anti-D | IgG | Distinct heavy and light bands | 1/64K | 15 Neat |
| **LHM77/64** | Anti-D | IgG | Distinct heavy and light bands | 1/1024 | 1 Neat |
| **LHM70/45** | Anti-D | IgG | Distinct heavy and light bands | 1/4096 | 5 Neat |
| **LHM59/19** | Anti-D | IgG | Distinct heavy and light bands | 1/16K | 165 Neat |
| **LHM169/80** | Anti-D | IgG | Distinct heavy and light bands | 1/64K | 206 Neat |
| **LDM1** | Anti-D | IgM | Heavy and light bands not obvious | 1/16 | 1.4 Neat |
| **LDM77/64** | Anti-D | IgM | Heavy and light bands not obvious | 0 | 0.3 Neat |
| **ESD1M** | Anti-D | IgM | Distinct heavy and light bands | 1/8 | 0.6 Neat |
| **H48** | Anti-c | IgM | Distinct heavy and light bands | 1/1000 | 10 Neat |
| **DEM1** | Anti-E | IgM | Heavy and light bands well resolved | 1/16 | 6 Neat |

Table 1

Table 2.

| | Excitation wavelength (nm) | Detection wavelength (nm) |
|---|---|---|
| Case 488/1 | 488 | 530 |
| Case 543/1 | 543 | 570 |
| Case 543/2 | 543 | 578 |
| Case 543/3 | 543 | 585 |

## Legends to figures

**Figure 1**
Box and whisker plots comparing the intensity of flurophores spotted on gold and epoxy-silane coated glass slides. The box represents 75 % of the values with the bar within the box representing the median. The whiskers represent the limit of the further 25 % of spot values. Outliers are represented as bars beyond the whiskers. a – A fluorescein labelled oligonucleotide spotted on gold and epoxy silane slides and b – Fluorescein labelled erythrocytes on gold and epoxy-silane coated slides.

**Figure 2**
Blood typing of cells with a single cell antigen using a microarray. Dark Grey columns represent gold-coated slides and Light Grey columns represent epoxy-silane coated slides. Values are the median of 3 replicate slides, bars represent standard errors. a – A1, b – B1, c – A2 and d – O.

**Figure 3**
Blood typing of cells with a mixed population of surface antigens using a microarray. Dark Grey columns represent gold-coated slides and Light Grey columns represent epoxy-silane coated slides. Values are the median of 3 replicate slides, bars represent standard errors. a – A1BRhD, b – A2BRhD, c – BRhD, d – A1RhD, e – ORhD, f – AxRhd

**Figure 4**
Differentiation of peptide antigens using an array of Anti- c,D and E antibodies. Dark Grey columns represent reactions with R1R1 erythrocytes and Light Grey columns represent r"r" erythrocytes. Values are the median of 3 replicate slides, bars represent standard errors.

**Figure 5**
Dependence of label-free erythrocyte phenotyping on sample pre-dilution. Values are the median of 3 replicate slides, bars represent standard errors.

**Figure 6**
The effect of microarray scanner settings on label-free signal to noise ratio (S/N)

**Table 1**
Comparison of antibody characteristics.

**Table 2**
Wavelengths used for comparison of scanner settings

## APPENDIX 5. Presentations Given Related to this Thesis

Protein Microarrays with Diagnostic Potential. Young Scientist Symposium, British Blood Transfusion Society Annual Meeting & Conference. Edinburgh, 6th September 2002.

New Technologies in Blood Transfusion Testing. Alba Bioscience, 2004.

Protein Microarrays and Their Potential Use in Blood Donation Testing. National Science Laboratory, Scottish National Blood Transfusion Service. April 23rd 2004.

## APPENDIX 6. Conferences and Courses Attended

Scotblood, Annual Scottish National Blood Transfusion Service Meeting. Stirling, 1st-3rd June 2001

Scotblood, Annual Scottish National Blood Transfusion Service Meeting. Stirling, 7th-9th June 2002

British Blood Transfusion Society Annual Meeting & Conference. Edinburgh, 5th-8th September 2002

Scotblood, Annual Scottish National Blood Transfusion Service Meeting. Stirling, 6th-8th June 2003

British Blood Transfusion Society Annual Meeting & Conference. Manchester, 2nd-5th October 2003

Scotblood, Annual Scottish National Blood Transfusion Service Meeting. Stirling, 4th-6th June 2004

International Society of Blood Transfusion Annual Meeting. Edinburgh, July 2004.

Thesis Workshop. Transferable Skills Programmed for PhD Students. College of Science and Engineering, College of Medicine and Veterinary Medicine. Swann Building, Kings Buildings. Dr Josephine Pemberton & Dr David Williams. November 2004.

Scotblood, Annual Scottish National Blood Transfusion Service Meeting. Stirling, 3rd-5th June 2005

International Society of Blood Transfusion Annual Meeting. Athens 2005.